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ABOUT COVER

Editorial board member of *World Journal of Clinical Oncology*, Dr. Fabrício Freire de Melo is a Professor at the Multidisciplinary Institute of Health of the Federal University of Bahia, Brazil. He undertook his postgraduate training at the Faculty of Medicine of the Federal University of Minas Gerais, where he received his Master's degree (2007) and PhD (2011), and completed his Postdoctoral Fellowship (2013) in Microbiology. His ongoing research interests involve the host-pathogen interactions in *Helicobacter pylori* gastric infection and the features associated with development of duodenal ulcer and gastric cancer. In addition, he has been investigating diagnostic methods for and the immune response in COVID-19. Currently, he serves as Research and Extension Coordinator of the Multidisciplinary Institute of Health of the Federal University of Bahia. (L-Editor: Filipodia)

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FRONTIER

Tumor-specific lytic path "hyperploid progression mediated death": Resolving side effects through targeting retinoblastoma or p53 mutant

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Author contributions: Hong F has worked on cancer biology, genetics, and pharmacology research; Castro M was involved in therapeutics research; Linse K performed molecular modeling to analyze the 3-dimensional structure of proteins; Hong F, Castro M, and Linse K meet the criteria for authorship established by the International Committee of Medical Journal Editors and verify the validity of the results reported.

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Abstract

A major advance was made to reduce the side effects of cancer therapy *via* the elucidation of the tumor-specific lytic path "hyperploid progression-mediated death" targeting retinoblastoma (Rb) or *p53*-mutants defective in G1 DNA damage checkpoint. The genetic basis of human cancers was uncovered through the cloning of the tumor suppressor *Rb* gene. It encodes a nuclear DNA-binding protein whose self-interaction is regulated by cyclin-dependent kinases. A 3D-structure of Rb dimer is shown, confirming its multimeric status. Rb assumes a central role in cell cycle regulation and the "Rb pathway" is universally inactivated in human cancers. Hyperploidy refers to a state in which cells contain one or more extra chromosomes. Hyperploid progression occurs due to continued cell-cycling without cytokinesis in G1 checkpoint-defective cancer cells. The evidence for the triggering of hyperploid progression-mediated death in RB-mutant human retinoblastoma cells is shown. Hence, the very genetic mutation that predisposes to cancer can be exploited to induce lethality. The discovery helped to establish the principle of targeted cytotoxic cancer therapy at the mechanistic level. By triggering the lytic path, targeted therapy with tumor specificity at the genetic level can be developed. It sets the stage for systematically eliminating side effects for cytotoxic cancer therapy.

Key Words: Retinoblastoma protein; P53 protein; Cancer; Checkpoint; Taxol; Tumor-specific lytic path

Core Tip: Side effect remains a major impediment to achieving a cure. An important

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advance has been made to establish the principle of cytotoxic cancer therapy at the mechanistic level. It concerns the discovery of the tumor-specific lytic path "hyperploid progression mediated death" targeting retinoblastoma (or p53) mutants defective in G1 DNA damage checkpoint. By triggering the lytic path, tumor specificity can be achieved at the genetic level for cytotoxic drugs.

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BIOGRAPHY

Frank-Un Hong (a.k.a. Frank Un, Frank D. Hong) is an established cancer researcher and research scientist at Bio-synthesis, Inc. While serving as a consultant in Beckman Research Institute of City of Hope National Medical Center, it became the top cancer center in the western half of the United States. He also served as a consultant in the highly regarded School of Pharmacy at the University of Southern California. As faculty at the top-ranked University of Texas M. D. Anderson Cancer Center, he contributed editorially for research journals, *e.g.* Gene therapy and Molecular Biology, Mini-reviews in Medical Chemistry. His works have been presented at the University of California at Los Angeles Keystone conference, National Eye Institute conference, the American Association of Cancer Research symposium, esteemed Cold Spring Harbor Symposium on Quantitative Biology on several occasions, and covered by news media.

Of historic relevance, T. Svedberg, who invented ultracentrifuge (Nobel prize 1926), assisted A. Tiselius in developing electrophoresis (Nobel prize 1948) and supported the "Brownian motion" proposed by A. Einstein (Nobel prize 1921). At the University of Uppsala (Sweden), Svedberg was succeeded by S. Claesson (Nobel committee member for chemistry), who also taught at the Baylor University, under whom F. Hong studied physical chemistry in 1980. He also studied polymer chemistry under M. Dole in 1981, who discovered the "Dole effect", which led to electrospray ionization mass spectrometry (Nobel prize 2002, J. Fenn, K. Tanaka). In 1979-1980, F. Hong worked on the organic synthesis of oligodeoxynucleotides using the phosphoramidite chemistry with W. Lunsford, a former colleague of W. Letsinger—a field to which H. Khorana (Nobel prize 1968, tRNAs encode amino acids) contributed. In 1983-1984, at the American Bio-Nuclear of F. Hoffmann-La Roche, F. Hong achieved the chemical synthesis of protected purine deoxynucleotide for oligonucleotide synthesis.

In 1981-1982, at the Johns Hopkins University, F. Hong worked on the cloning of phosphate transferase system-encoding genes with its discoverer S. Roseman, who uncovered that phosphate transferase system governs diauxie, whose prior interpretation by J. Monod led to the ''operon'' concept (Nobel prize 1965). In 1982, F. Hong worked on "transdetermination" with A. Shearn, which is related to altered embryonic phenotype caused by the mutant homeotic gene (Nobel prize 1995, E. Lewis, C. Nusslein-Volhard, E. Wieschaus).

In 1985, at the Salk Institute founded by J. Salk (developed polio vaccine), F. Hong determined the genomic sequence of the avian infectious bronchitis virus strain M41-positive-sense single-stranded RNA coronavirus (belongs to the identical family as SARS or coronavirus disease 2019 coronavirus but distinct genus). This was done to identify the neutralizing antibody epitopes on spike protein with W. Spaan (University of Utrecht, The Netherlands), which was reported in Virus Research[1]. Vaccines prepared for the subsequent epidemic by "SARS coronavirus" likewise target the spike protein. In 1984-1985, F. Hong worked on the intracellular transport mechanism (of Vesicular stomatitis virus's G protein) with J. Rose, a former colleague of D. Baltimore (Nobel prize 1975, reverse transcriptase), which is implicated in diabetes or mental disorder (Nobel prize 2013, Scheckman, Sudhof, Rothman).

In 1985-1986, at the scripps research institute, F. Hong cloned/determined the first eukaryotic cDNA sequence of human muscle 6-phosphofructokinase gene with S. Vora of E. Beutler's group (parent of B. Beutler, Nobel prize 2011, Toll-like receptor),

which was reported in Biochem. Biophys. Res. Comm[2]. Phosphofructokinase-M is linked to Tarui disease, cancer metabolism, and represents one of the earliest susceptibility genes to be identified for human heart disease (cardiac hypertrophy).

In 1986-1987, at the University of California at San Diego, F. Hong identified/cloned human retinoblastoma susceptibility gene Rb, proving the genetic basis of human cancers, which was published in Science[3]. This work was done with W. Lee, a former colleague of P. Duesberg (discovered v-Src oncogene of Rous sarcoma virus, which led to c-Src discovery; Nobel prize 1989, M Bishop, H Varmus). Mutation in tumor suppressor gene *RB* was also found in osteosarcoma, breast cancer, small cell lung cancer, colon cancer, and glioma. It inspired the cloning of other tumor suppressor genes: Example, p53, breast cancer 1, phosphatase and tensin homolog, neurofibromatosis type 1, Wilms' tumour 1.

In 1986-1987, F. Hong determined the cDNA sequence of human RB [the N-terminus that are GC-rich *via* the Maxam-Gilbert method[4] and the C-terminal region *via* Sanger's chain termination method[3], which contained cyclin-dependent kinase (CDK) phosphorylation motifs. It led to the subsequent identification of CDK4/6 as the regulator of Rb, which governs cell cycle progression across "restriction point" at the G1 phase. This was followed by the development of the CDK4/6 inhibitor (example, palbociclib by Pfizer Inc.), which was approved by the United States Food and Drug Administration to treat advanced-stage breast cancer.

In 1987, F. Hong discovered Rb's DNA binding property that revealed Rb's role in regulating transcription or DNA replication, which was reported in Nature[4]. It instilled the view that Rb may function as a transcription factor—example, by interacting with E2F or other associating proteins. Subsequently, he documented a stable complex formed by purified Rb protein and double-stranded DNA, which indicated that Rb interacts with DNA directly[5].

In 1987-1988, he observed that SV40 promoter-driven Rb overexpression causes cells to arrest as enlarged cells in cell culture (unpublished), which preceded the subsequent discovery of Rb's function in DNA damage checkpoint in G1 or S phase.

In 1988-1989, F. Hong identified/characterized the *Rb* gene promoter and found its regulatory elements reminiscent of "housekeeping" genes, which was reported in Proc Natl. Acad. of Sci. United States[6]. In 1990, he reported the discovery of the first *Rb* mutation in human prostate cancer, *i.e.* defective promoter, which was published in Proc Natl. Acad. of Sci. United States[7]. It led to the finding that mutant Rb triples the mortality risk of prostate cancer patients and that Rb loss confers resistance to androgen receptor antagonizing therapy.

In 1989, he found a similarity between the Rb polypeptide sequence and the neurofilament subunit NF-L, indicating that Rb may function in the structural aspect of chromosomes, which was reported in Bioscience Report[8]. In 1990-1991, he discovered Rb's oligomerizing property, which was published in the Journal of Biological Chemistry[9]. It suggested that Rb may form a higher-ordered structure like nuclear matrix ("corral hypothesis" presented at Cold spring Harbor Symposium on Quantitative Biology in 1991[10] and 1994[11] with the latter organized by J. Watson; Nobel prize 1962, double helix) to assume its function—*i.e.* modulating chromatin to affect DNA condensation, histone modification, heterochromatin, DNA replication. It led to the finding that Rb's N-terminus interacts with its C-terminus, which was subsequently confirmed. Further, it revealed that CDKs negatively regulate the Rb-to-Rb self-interaction (presented at the 1994 Cold Spring Harbor Symposium on Quantitative Biology)[11].

In 1993, at the Fred Hutchinson Cancer Research Center, F. Hong worked on the human *APC* gene, whose inactivation causes familial adenomatous polyposis with its discoverer G. Joslyn, a former colleague of R. White (University of Utah).

In 1993-1995, at the Salk Institute, F. Hong worked on growth factors, Alzheimer's disease, and aging disorders with D. Schubert, a former colleague of J. Monod (Pasteur Institute, France).

In 1996-1997, F. Hong discovered the tumor-specific lytic path "hyperploid progression mediated death" targeting Rb or p53 mutant cancers defective in G1 DNA damage checkpoint while investigating the mechanism through which Taxol induces chromosomal aneuploidy in human brain cancer cells, which was published in Carcinogenesis[12]. This work was done with P. Nisen, a former colleague of S. Cohen (Nobel prize 1986, epidermal growth factor) who worked on gene therapy of brain cancer. In 2010, the events that led to its discovery were chronicled in the book, "Multiple Drug Resistance"[13]. The lytic path has been confirmed globally.

In 2005-2006, at the City of Hope National Medical Center's Beckman Research Institute, F. Hong discovered the mechanism through which the sensitivity of resistant cancers to the antimetabolite drug hydroxyurea could be restored by modulating the

Rb-associating transcription factor ICBP90 (UHRF1), which was reported in Anticancer Research[14]. In 2007, F. Hong reported the discovery that Rb protein mediates the cytotoxicity of the DNA crosslinking drug cisplatin in G1 DNA damage checkpoint-retaining human cancers in Anticancer Drugs[15].

In 2015-2018, at the University of Southern California, F. Hong worked on the role of monoamine oxidase (MAO), which degrades serotonin (5-HT) and other neurotransmitters, in human cancer with J. Shih (part of a delegation sponsored by the United States National Academy of Science to open diplomatic relation with China on the scientific front during the Nixon administration), who discovered MAO isoenzymes A and B *via* cloning, the role of MAO in autism, and the genetic regulation of behavior.

INTRODUCTION

Advances in medicine come slowly. The discipline of cancer therapy is no exception as there has been little increase in survival within the last fifty years for certain human cancers despite extensive research. One of the key issues standing in the way of achieving a cure is side effects as it undermines dose escalation or prolonged treatment. The problem is well documented for chemotherapy, which nevertheless remains one of the major treatment modalities. This is because chemotherapeutics can induce cancer cell death (cytotoxic) rather than merely arrest cycling cells (cytostatic), which represents an important therapeutic asset. Here, a major advance made to solve the problem of side effects is described.

Chemoprevention

The basic tenet underlying chemoprevention is to reverse the course of cellular changes occurring during a normal to cancerous state transition through the pharmacological intervention. In several cases, the precise stages that a normal, well-differentiated cell undergoes to become cancerous have been well documented. One such example is colon cancer, where the transition of a normal cell through the "polyp" stage in route to becoming cancerous has been clearly documented. In the case of an intestinal tumor, this scenario has been confirmed using an Apc (adenomatous polyposis coli)-gene inactivated murine model[16]. By understanding the molecular mechanism underlying this process, several targets can be identified for pharmacological intervention.

In the case of colon carcinogenesis, overexpression of the gene encoding cyclooxygenase (COX), which is responsible for the production of prostaglandin from arachidonic acid, is an early and central event. This has led to the development of COX-inhibiting nonsteroidal anti-inflammatory drugs such as sulindac, or more selective COX-2-specific drugs.

In the case of breast carcinogenesis, the estrogen pathway has been targeted for chemoprevention given estrogen's role as a tumor promoter. Tamoxifen is an analog of estrogen used for suppressing mammary carcinogenesis. Raloxifene represents another agent with a similar potential. Development of selective estrogen receptor modulators, which function as estrogen agonists in tissues where estrogen is beneficial yet as antagonists in tissues where estrogen may promote carcinogenesis (breast, uterus, ovary) remains the goal.

For cancers of the head and neck as well as the lung, the retinoid pathway has been targeted for chemoprevention. Retinoids are essential for proper differentiation of lung and upper airway epithelium, and the loss of retinoic acid receptor (RAR) has been associated with premalignant lesions of the oral epithelium and the formation of lung cancer[17]. Consequently, (nuclear) receptors for retinoids represent critical molecular targets for chemoprevention. The nuclear receptors function as transcription factors for specific genes, whose promoters contain their responsive elements. Administration of 9-cis-retinoic acid, which represents an agonist for all 6 types of retinoid receptors, has been shown to restore the expression of RAR, and reverse the development of cancerous lesions[18]. Those binding to retinoid X receptors but not to RARs such as targretin may represent more selective chemopreventive agents for these cancers[19].

Chemotherapy

The majority of previously developed anti-cancer drugs were designed to interfere with various aspects of DNA metabolism. The underlying rationale is that interfering with the propagation of genetic material will undermine the continuous cell division necessary for tumor formation. The targeted areas of DNA metabolism include DNA

replication, DNA repair, chromosome segregation, DNA mutagenesis, *etc*.

One class of such anti-cancer drugs includes agents that form noncovalent complexes with DNA. These include anthracyclines, mitoxantrone, dactinomycin, bleomycin, and plicamycin. Doxorubicin (adriamycin) and daunorubicin, isolated from different species of *streptomyces*, belong to the category of anthracyclines. In the case of doxorubicin, it intercalates with double-stranded DNA and causes local uncoiling. This happens due to the separation of the stacked bases by the intercalated doxorubicin molecule. Doxorubicin treatment of cells results in the cleavage of their DNA. Cleavage of the intracellular double-stranded DNA is thought to occur due to the inhibition of topoisomerase II. Cleavage of single-stranded DNA is thought to occur due to the formation of the drug-Fe-DNA complex, which induces the generation of hydroxyl radicals due to hydrogen peroxide[20].

In contrast, cisplatin belongs to a different class of anti-cancer drugs that interact directly with DNA, through forming crosslinks. The ability of cisplatin to form adducts with DNA has been exploited for the treatment of head and neck cancer.

Other types of anticancer drugs that do not interact with DNA directly, yet affect its metabolism indirectly, include methotrexate, which inhibits the synthesis of nucleotides necessary for DNA replication. They also include Taxol and vincristine, the antimicrotubule drugs that interfere with the DNA metabolism at the level of sister chromatid segregation (which occurs during metaphase-to-anaphase transition of mitosis) by blocking the formation of mitotic spindles.

Certain anticancer drugs such as etoposide function by inhibiting enzymes involved in the repair of DNA breaks, such as topoisomerase II.

The unresolved issue of side effects

The occurrence of side effects continues to pose a problem for chemoprevention and chemotherapy. Side effects associated with chemopreventive drugs include the following. In the case of the COX-inhibitors, their nonselective aspect can lead to serious side effects including gastrointestinal ulceration and bleeding. The side effects have mainly been attributed to the inhibition of the COX-1 enzyme, which is involved in generating prostaglandin. Prostaglandin functions to maintain stomach lining integrity, regulate blood flow within the kidneys, and balance platelet function. This has prompted the development of more selective drugs, *i.e.* inhibitors of COX-2 enzymes whose level increases in response to diet, stress, and injury. However, treatment with COX-2 inhibitors has been associated with an increased risk for heart attack and stroke[21].

The use of tamoxifen to treat breast cancer has been associated with the occurrence of endometrial cancer and thromboembolism. Raloxifene, an estrogenic agent that maintains bone mass in postmenopausal women to prevent osteoporosis, is used for breast cancer chemoprevention and has a similar degree of risk as tamoxifen for developing thromboembolism[22].

The use of retinoids has been associated with various toxicities including skin dryness, cheilitis, hypertriglyceridemia, and conjunctivitis.

For the majority of drugs used in chemotherapy, side effects remain a major problem. As most currently used drugs were designed to interfere with various aspects of DNA metabolism necessary for rapidly replicating cancer cells, it undoubtedly has a negative effect on normal cells that also divide frequently, ex. white blood cells. The lack of tumor-specificity may result in side effects including weight loss, infection (due to decreased immunity), fatigue, and pain.

Among various main-line therapies, chemotherapy utilizing Taxol has emerged as one of the most potent treatments for breast, ovarian, and lung cancers. But treatment with Taxol results in various side effects, including dyspnea with bronchospasm, urticaria, hypotension, neutropenia, and peripheral neuropathy[23].

For the crosslinking drug cisplatin, nephrotoxicity is its chief dose-limiting side effect[24]. For anthracyclines like doxorubicin, side effects include cardiac toxicity, bone marrow suppression, and gastrointestinal and hepatic effects[25]. The side effects, resulting from their lack of tumor-specificity, remain a major hurdle as it undermines the effort to attain the dose necessary for tumor destruction.

Another significant area of concern for DNA-targeting anticancer drugs is their mutagenic nature. Because drugs such as cisplatin or alkylating drugs interact directly with DNA, its mutagenic nature can contribute to the development of secondary malignancies.

Tumor-specific lytic path provides solution through genetic specificity

The problem facing current cancer therapy is not the lack of cytotoxic drugs per se but the lack of cytotoxic drugs that are tumor-specific. Indeed, the vast majority of drugs

administered today as part of chemotherapy are capable of lysing cancer cells. Yet, upon administration, their propensity to cause side effects presents a major impediment as it incurs a debilitating effect and preempts dose-escalation necessary to eliminate recurrent cancers. The prevailing view is that the cytotoxicity results from their ability to interfere with cell cycle progression—hence, leading to the death of cancer cells as well as normal cells that divide.

Yet, this view may have oversimplified the scenario. First, pharmacologically, many of these drugs were isolated based on their ability to lyse cancer cells, not normal cells. The key amongst them is Taxol, which was isolated after screening various plant extracts for a tumor suppressing potential. Second, physiologically, many of these drugs represent a part of the host's defense repertoire against invading microorganisms—hence, were not designed to inflict damage on normal cells. Third, clinically, these drugs can achieve a cure in a minor subset of cancer patients despite that both the responders and nonresponders suffer side effects. These discrepancies have led to the speculation that the anti-tumor mechanism of these drugs may be distinct from the mechanism underlying their side effects, which ultimately led to the elucidation of a tumor-specific lytic path.

During 1996-1999, while working on the mechanism through which antimicrotubule drugs like taxol induce chromosomal aneuploidy, a tumor-specific lytic path targeting G1 checkpoint defective human cancers was discovered by serendipity (Figure 1). The tumor-specific lytic path ''hyperploid progression mediated death'' is specific for RB or p53 genetic mutants that are defective in DNA damage checkpoint for the G1 phase[12]. Through its elucidation, the principle of tumor-specific cytotoxic therapy, which is based on the genetics of human cancer, was established. Previously, the lytic path was mentioned in a biological context[26]. Subsequently, the events that led to its discovery were chronicled in the book "Multiple Drug Resistance"[13]. More significantly, for cancer therapy, it provided a mechanistic framework for developing cytotoxic drugs devoid of side effects by conferring tumor specificity at the genetic level.

Universal inactivation of the Rb pathway in human cancers

The genetic basis of human cancers was elucidated through the identification of the prototypic tumor suppressor gene Rb, whose inactivation predisposes to the development of retinoblastoma[27]. The underlying genetic mechanism was proposed by A. Knudsen, who suggested that its dominant pattern of inheritance could be explained through the late inactivation of the remaining wild-type allele in a hereditary case harboring a mutant allele[28]. It also suggested that the sporadic cases may take a longer period to develop tumors due to the time it takes to acquire mutations in both wild-type alleles.

The retinoblastoma susceptibility gene was identified through molecular cloning, which was conducted in several laboratories[3,29,30]. The human *Rb* gene consists of 27 exons and the germline and somatic mutations may occur throughout its coding region as well as the promoter[6,31]. The delineation of the RB gene structure has significantly advanced our ability to manage retinoblastoma clinically through genetic diagnosis[32]. Subsequent investigations documented the occurrence of *Rb* gene mutation in various human cancer types including breast cancer, prostate cancer, osteosarcoma, non-small cell lung cancer (> 80%), and brain cancer[6,33-35].

Rb protein (also known as RB1) is a key component of mammalian DNA damage checkpoint that monitors the integrity of DNA and blocks cell cycle progression past the G1 (or S) phase in the event of DNA damage. The arrest at G1 is critical as it renders time to repair damaged DNA and avoid replicating mutated DNA. The step mediated by Rb represents a focal point where growth regulatory signals transduced through mitogen-activated protein kinase/ extracellular signal-regulated kinase or phosphatase and tensin homolog/AKT pathway as well as mitogenic signals initiated by epidermal growth factor receptor or ERBB2 (also known as Her2) converge to modulate cell proliferation--hence, giving rise to the concept that Rb represents the center of cell cycle control.

Nevertheless, the exact molecular mechanism through which Rb executes G1 arrest remains incompletely understood[36]. The binding of Rb to DNA-affinity columns suggested that Rb's intracellular function may be regulatory in nature, which was confirmed by its role in regulating gene transcription[4,37,38].

Critical insight into the Rb function was obtained upon uncovering its propensity to form oligomers through self-interaction[8,9]. The observation that cellular Rb exists as a dimer in the cell lysate confirmed its oligomerization potential[10]. A 3-dimensional model showing Rb protein dimer as observed in the asymmetric crystal unit for Rb^{PL-P} is shown (Figure 2). The crystal structure (2.0 Å) of an Rb construct containing "pocket

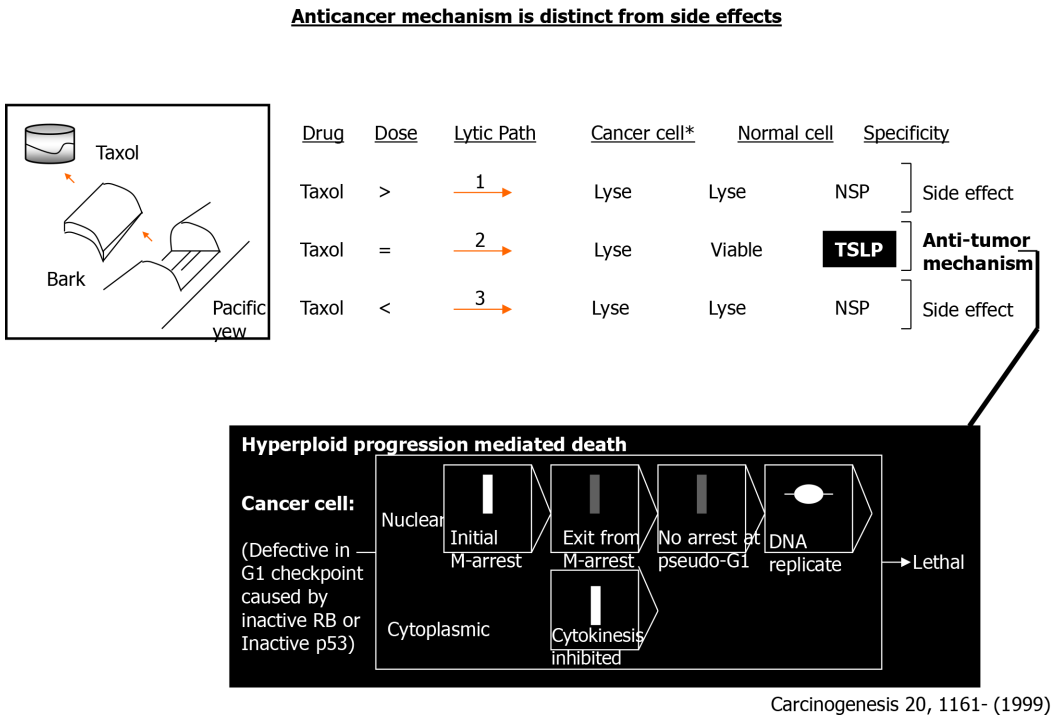

Figure 1 Side effects are distinct from the anticancer mechanism. An exemplary case concerning taxol is depicted. TSLP: Tumor-specific lytic path; >: Greater than microbutule-disruping dose; =: Equivalent to microtubule-disrupting dose; <: Lesser than microtubule-disrupting dose; NSP: Nonspecific.

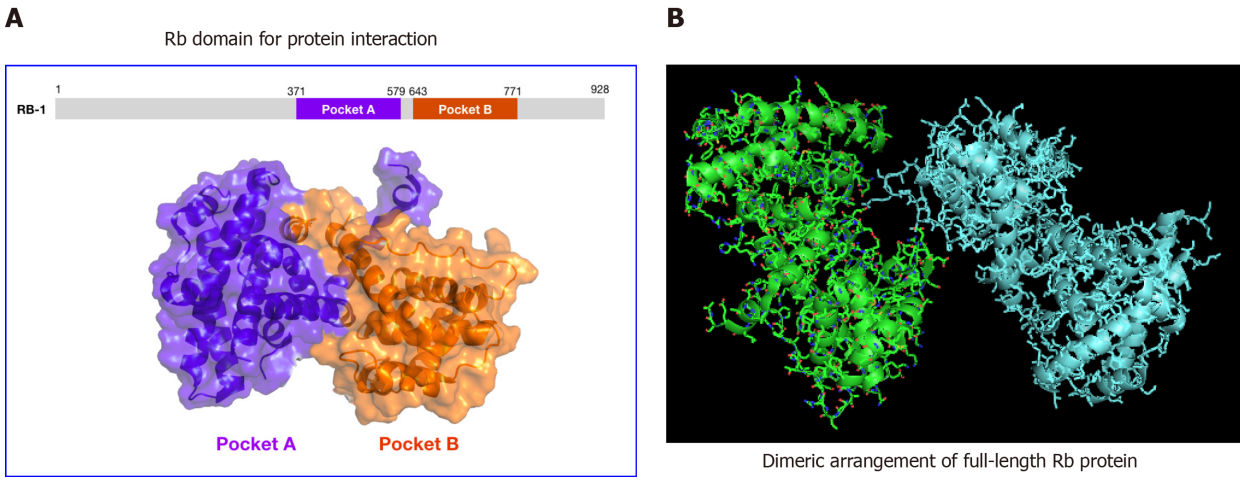

Figure 2 Structural analysis of human retinoblastoma protein. A: 3D structure of the inactive retinoblastoma protein pocket domain of retinoblastoma-1 (PDB ID: 4ELL). The model was generated using PyMol molecular graphics system, version 1.2r3pre, Schrödinger, LLC. B: Structures (or structural model) of phosphorylated retinoblastoma. The model shows the protein dimer as observed in the asymmetric crystal unit for retinoblastoma^{PL-P}. The structural coordinates for PDB ID 4ELL were downloaded from the NIH protein database. PyMol was used to re-enter the image of the model as shown. Rb: Retinoblastoma.

domain" with the phosphoserine-mimetic (S608E) and a shortened RbPL (large loop within the pocket domain) was solved by Burke *et al*[39] *via* crystallizing Rb^{PL-P} (representing Rb380-787Δ616-642/S608E/S612A/S780A) that binds E2F^{TD} (transactivating domain of E2F) with a lesser affinity, suggesting that the glutamate substitution mimics phosphorylated S608. Rb's post-translational modification *via* phosphorylation, which was uncovered through the identification of cyclin-dependent kinase recognition motifs in the Rb polypeptide sequence, was shown to negatively regulate Rb self-interaction[11]. These findings suggested that Rb may form a higher-ordered structure *in vivo* to execute G1 arrest.

The central role of Rb in regulating cell cycling stems from the post-translational modification of Rb by various cyclin-dependent kinases, which occurs progressively as cells transit from the G1 to the M phase. These include Cdk4 and Cdk6 in the G1 phase

and Cdk2 in the S phase whose activities require associating with distinct cyclins. The activities of specific Cdks are further modulated through complexing with other factors such as $p14^{Art}$ and p16, with the latter representing a distinct tumor suppressor. The activity of Cdk2 is inhibited by $p21^{Cip1/Waf1}$, whose transcription is regulated by p53, which is mutated in nearly 50% of all human cancers. Additionally, the oncogenic proteins encoded by viruses such as papillomavirus and adenovirus target Rb to promote cell proliferation. As many as 200 cellular proteins may interact with Rb, including E2F regulating the transcription of genes involved in S phase activities that bind to the pocket domain. The above "Rb pathway" is inactivated in nearly all human cancers.

Triggering of hyperploid progression mediated death in Rb mutant human cancer cells

Hyperploid progression occurs due to continued cell cycle progression without cytokinesis. Briefly, disruption of the mitotic spindle by antimicrotubule drugs activates spindle checkpoint, a component of M phase DNA damage checkpoint, to induce M arrest. After a transient M-arrest, the treated cells re-enter the cell cycle (due to mitotic slippage) without cytokinesis to eventually become re-arrested at a "G1-like" phase in Rb-retaining cells. In cells lacking Rb, however, the treated cells continue with DNA replication, resulting in hyperploidy. Continued treatment with the antimicrotubule drug leads to the death of the hyperploid cells.

Experimental evidence for the triggering of hyperploid progression mediated death in RB-mutant human cancer cells is shown. WERI-1 is a human retinoblastoma cell line lacking both RB alleles[40]. Continued treatment of WERI-1 cells with the antimicrotubule drug nocodazole led to the death of hyperploid cells induced (Figure 3).

Triggering of hyperploid progression mediated death by antimicrotubule drugs

After the initial report by Hong *et al*[12] in July of 1999, multiple other reports followed providing additional evidence for hyperploid progression mediated death. The first category of such reports used antimicrotubule drugs as the inducer.

In August of the same year, Casenghi *et al*[41] of the University of La Sapienza in Italy reported the propensity of K562 cells lacking p53 to undergo hyperploid progression before dying following the nocodazole treatment. In the report, polyploidization was confirmed *via* in situ hybridization using chromosome-specific pericentromeric probes. Their exit after a transient M arrest was confirmed by assessing the cyclin B1 or MPM-2 level and the re-initiation of DNA replication was detected by flow cytometric analysis of bromodeoxyuridine (BrdUrd)-incorporated cells.

In September of the same year, Verdoodt *et al*[42] of Vrije Universiteit Brussel in Belgium reported that a positive correlation exists between the extent of polyploidization induced by nocodazole and the level of apoptosis.

In 2001, Tsuiki *et al*[43] of Kumamoto University in Japan reported that U251MG cells containing mutant p53 undergo hyperploid progression (> 4N or 8N peak appeared) before dying (sub-2N or 0-1N peak detected) as monitored by flow cytometry. The authors also showed that enhancing hyperploid progression through the broad-range protein kinase inhibitor Staurosporine causes a greater extent of lethality in U251MG cells.

In the same year, Cassinelli *et al*[44] of instituto Nazionale per lo studio e la Cura dei tumori in Italy also reported that treatment of p53-mutant human ovarian cancer IGROV-1/Pt1 cells, p53-deficient human prostate carcinoma P3 cells, or Saos-2 with taxol or its analog integrated digital network 5109 led to hyperploid progression and death.

In a separate report by Lanzi *et al*[45] of Instituto Nazionale per lo studio e la Cura dei tumori in Italy, the death of PC3 cells undergoing hyperploid progression following taxol treatment was directly shown using the TUNEL/PI double-staining method.

In 2002, Emory University reported that altering microtubule by noscapine caused murine melanoma B16S9 cells to undergo hyperploid progression before dying.

In 2012, Qi *et al*[46] of Showa Pharmaceutical University in Japan showed that pseudolaric acid B induces hyperploid progression, resulting in mitotic catastrophe before apoptosis in murine fibrosarcoma L929 cells. Pseudolaric acid B is a microtubule destabilizing agent found in the bark of pseudolarix kaempferi Gorden (*pinaceae*) tree in central China, which was used for treating fungal infection.

Triggering of hyperploid progression mediated death by alternate therapeutics

The second category of reports used antimitotic agents other than antimicrotubule

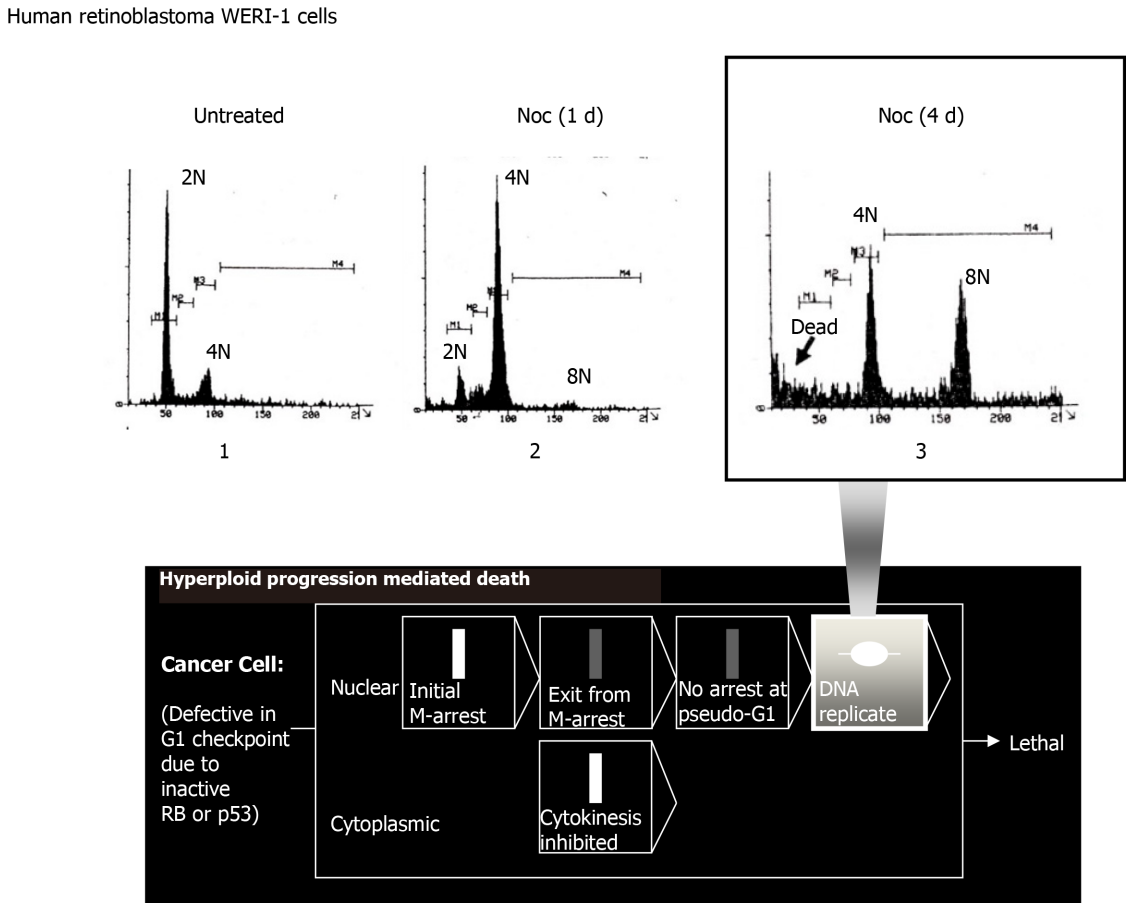

Figure 3 Nocodazole triggers hyperploid progression mediated death in retinoblastoma-mutant WERI-1 retinoblastoma cells. A DNA content flow cytometric histogram of WERI-1 cells continuously treated with nocodazole (Noc; 0.415 μmol/L) for the duration (1 d, 24 h; 4 d, 96 h) is shown. Cells (5 × 10^5) were treated as indicated, harvested using trypsin and fixed in 70% ethanol. To determine DNA content per cell, propidium (100 Kunitz units/mL) and RNase A (50 μg/mL) were added and assayed using Beckton Dickinson flow cytometry. Persistent treatment with nocodazole led to the death of hyperploid cells induced. RB: Retinoblastoma.

drugs as the inducer of hyperploid progression mediated death. In 2003, Ditchfield *et al*[47] of the University of Manchester and AstraZeneca Pharmaceuticals in England reported that ZM447439-treated HeLa cells undergo hyperploid progression and die. ZM447439 is an inhibitor of Aurora B kinase.

In 2004, Harrington *et al*[48] of Vertex Pharmaceuticals in England reported that treatment of HeLa cells or human breast cancer MCF-7 cells with VX-680 (tozasertib), which targets aurora kinase (see below) causes them to undergo hyperploid progression before dying. The exit from M phase of VX-680 treated cancer cells were monitored by assessing the cyclin B1 level. DNA replication occurring without the prior cytokinesis, leading to > 4N cells, was also described.

In the same year, Gizatullin *et al*[49] of Dana Farber Cancer Institute at Harvard Medical School reported that VX-680 triggers hyperploid progression before death in human non-small cell lung cancer A549 cells with a deficient level of p53-induced $p21^{cip1/waf1}$. They also were able to document that cells undergoing hyperploid progression were dying *via* apoptosis[49].

In 2005, Tao *et al*[50] of Merck Research Laboratories reported that treating human ovarian cancer A2780 cells with KSP-IA causes hyperploid progression and death. KSP-IA is an inhibitor of Eg5, a member of the kinesin-5 family that plays a critical role in chromosome segregation by maintaining spindle bipolarity. Eg5 functions as a molecular motor that slides along microtubule tracks within cells.

In 2006, Dijkhuis *et al*[51] of University Medical Center Gronigen in the Netherlands reported that treating with PDMP (D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) sensitizes neuroblastoma cells to paclitaxel by triggering hyperploidy. PDMP is an inhibitor of glucosylceramide synthase that suppresses

sphingolipid biosynthesis.

In the same year, Chin *et al*[52] of the DNAX Research Institute of Molecular and Cellular Biology Research Institute reported that treating M checkpoint-suppressed HeLa cells with monasterol causes hyperploid progression and death. Monasterol is an inhibitor of kinesin-5.

In 2007, New York Medical College reported that treatment of human prostate cancer PC-3, CWR22Rv1, or DU-145 cells with Reversine causes polyploidy *via* suppressing cyclin B or Cdk1, resulting in growth arrest.

In 2008, D'Alise *et al*[53] of the European Institute of Oncology in Italy reported that the synthetic purine reversine inhibits the aurora kinase and induces hyperploid progression and death in human colon cancer HCT116 cells.

In the same year, Hauf *et al*[54] of Research Institute of Molecular Pathology in Austria reported that treating HeLa cells with hesperadin, which also targets aurora kinase, causes them to undergo hyperploid progression before dying. Flow cytometry showed the emergence of hyperploidy peak (8N and 16N) preceding the appearance of dead cells[54].

In 2018, Cheng *et al*[55] of Peking University in China demonstrated that the treatment of human renal carcinoma cells with Reversine led to polyploidy formation and caspase-dependent cell death.

Current pharmacological approaches targeting RB or p53 mutants

In the case of p53, its mutant form may impart oncogenic properties to the affected cell. Nevertheless, various molecularly targeted approaches for treating p53-mutant cancers have been developed[56]. For RB, its loss is associated with enhanced efficacy for ionizing radiation therapy, chemotherapeutics such as cisplatin or adriamycin[57].

In 2018, Knudsen *et al*[57] of Thomas Jefferson University screened for therapeutic agents that may exhibit synthetic lethality with Rb loss. They found that drugs inhibiting checkpoint kinase1 (CHK1) or polo-like kinase 1 (PLK1) kinase exhibit greater lethality in *Rb* mutant tumors. The finding raises the potential of treating triple-negative breast cancers with dysfunctional Rb[58]. CHK1 encodes a serine/threonine kinase whose activation initiates cell cycle arrest in response to DNA damage during S, G2, and M phases. PLK1 is a serine/threonine kinase that activates cdc25C, which in turn activates cyclin B/cdc2 complex through dephosphorylation, as well as anaphase-promoting complex to transit from G2 to M phase.

In 2019, Gomaa *et al*[59] of the University of Miami Miller School of Medicine showed that reconstituting the microRNA, miR-4715--3p, reduced aurora kinase A level in MKN45 gastric cancer cells, resulting in chromosome polyploidy and cell death. miR-4715--3p is an epigenetic regulator that downregulates aurora kinase A expression by binding to the 3'-untranslated region of its mRNA for degradation[59].

In the same year, Sun *et al*[60] of the National Cancer Institute demonstrated that genetically silencing INCENP (Inner centromere protein) in neuroblastoma cells induces polyploidy and apoptosis. The *INCENP* gene encodes a key scaffolding component of chromosomal passenger complex consisting of survivin, aurora kinase B, borealin, and INCENP, which oversees proper alignment and segregation of chromosomes and cytokinesis during mitosis.

Additionally, Zheng *et al*[61] of the University of Texas M. D. Anderson Cancer Center showed that treating lung cancer cells with a highly selective tyrosine threonine kinase inhibitor, CFI-402257, causes aneuploidy and apoptosis. TTK (also called Mps1) or tyrosine threonine kinase is a component of mammalian spindle assembly checkpoint, which is integral to maintaining chromosome integrity.

In 2020, Simon Serrano *et al*[62] of the Lund University in Sweden reported that inhibiting Mps1 kinase (mitotic kinase monopolar spindle 1) induces hyperploid progression mediated death in neuroblastoma cells. The mechanism of death in Mps1 inhibited cells involves transiting through polyploidization/aneuploidization before the onset of mitotic catastrophe[62].

Combination therapy targeting RB or p53 mutants

In 2010, Charité-Universitätsmedizin Berlin in Germany determined that combining the aurora kinase inhibitor ZM447439 significantly improves the antiproliferative effects of the chemotherapeutic drug cisplatin and streptozocin. For the study, the authors employed gastroenteropancreatic neuroendocrine tumor cells.

In 2017, Czech Academy of Sciences in the Czech Republic examined the effect of combining CHK1 kinase inhibitor SCH900776 with the DNA crosslinking drug cisplatin or platinum (IV)-LA-12 complexes in treating colon cancer. In p53 or p21 deficient cells, the combination therapy increased mitotic slippage, leading to polyploidy. Further, the delayed death caused by the drug combination in p53

deficient cells was accelerated by p21 deficiency[63].

In the same year, Bressy *et al*[64] of Université Paris-Saclay in France assessed the therapeutic efficacy of combining oncolytic adenovirus containing delta-24 deletion in the E1A gene with valproic acid, a histone deacetylase inhibitor for colon carcinoma. Previously, it was shown that E1A interacts with Rb; thus, the recombinant virus may selectively replicate in Rb-deficient cancer cells but not in normal cells expressing wild-type Rb. The co-treatment led to polyploidy with increased H2AX phosphorylation indicative of DNA damage, as well as elevated cell death[64].

In 2018, Kawakami *et al*[65] of the University of Texas M. D. Anderson Cancer Center reported that treating with CFI-400945, which inhibits polo-like kinase 4 regulating centriole duplication, causes polyploidy and mitotic defect, resulting in the death of lung cancer cells. Further, it was shown to cooperate with the Cdk2 inhibitor seliciclib[65].

In 2019, Gong *et al*[66] of Eli Lilly and Company showed that cell cycle inhibitors targeting aurora kinase B exhibit synthetic lethality with *Rb* mutant. LY3295668 is an inhibitor of Aurora kinase but exhibits with > 1000-fold selectivity against Aurora kinase B. Further, prolonged treatment was possible due to little toxicity against bone marrow[66].

A recent report in 2020 by Liu *et al*[67] of Kaohsiung Medical University in Taiwan showed that treating non-small-cell lung cancer cells with 4-HPPP [4-(4-(4-hydroxyphenoxy) phenoxy) phenol] caused polyploidy-specific cell death. The treatment resulted in cellular aneuploidization accompanied by the activation of double-strand DNA break markers such as Ataxia-telangiectasia-mutated and Rad3-related and gamma-H2AX. Previously, the phenoxyphenol derivatives have been suggested to sensitize the lung cancer cells to the topoisomerase inhibitor camptothecin by reducing the apoptosis-inducing threshold[68].

In the same year, Jemaà *et al*[69] of Lund University in Sweden described that treating tetraploid colon cancer cells with PLK1 inhibitor caused mitotic slippage, followed by apoptosis. Further, combining PLK1 inhibitor with vincristine or colchicine resulted in greater lethality, demonstrating a synergistic effect.

CONCLUSION

With the event of targeted therapy, an enormous capital has been spent by pharmaceutical industries on developing drugs that inhibit oncogenic signaling pathways. Equally staggering is the amount of federal funds allocated to assess their clinical efficacy. Yet, for the most part, benefits have been incremental. In the case of anti-ErbB2 drugs, only a modest gain in overall survival was achieved after combining with chemotherapy. With the PI3K inhibiting drugs, a similar outcome was observed upon combining with anti-hormone therapy for advanced-stage breast cancer. Besides, the cytostatic nature of these drugs may require perennial treatment, which may not be ideal as cancer cells could acquire genetic mutations to become resistant. Against this backdrop, the elucidation of "hyperploid progression mediated death" targeting *Rb* or *p53* mutants assumes greater significance as it provides an opportunity to develop cytotoxic drugs with tumor specificity at the genetic level, thereby eliminating side effects.

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REVIEW

Liquid biopsy in ovarian cancer: Catching the silent killer before it strikes

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Abstract

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy in the western world. The majority of women presenting with the disease are asymptomatic and it has been dubbed the "silent killer". To date there is no effective minimally invasive method of stratifying those with the disease or screening for the disease in the general population. Recent molecular and pathological discoveries, along with the advancement of scientific technology, means there is a real possibility of having disease-specific liquid biopsies available within the clinical environment in the near future. In this review we discuss these discoveries, particularly in relation to the most common and aggressive form of EOC, and their role in making this possibility a reality.

Key Words: Epithelial ovarian cancer; Molecular profile; Liquid biopsy; Circulating tumor DNA; Biomarker discovery; Precision medicine

Core Tip: Epithelial ovarian cancer (EOC), particularly high-grade serous carcinoma, is a gynaecological malignancy with a poor survival rate. Currently there is no effective disease-specific biomarker, which could improve detection rates and treatment algorithms, for any of the EOC types - this is an area of unmet clinical need. Circulating tumour DNA (ctDNA) analysis has emerged as a potential blood-based

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"liquid biopsy" for early detection, diagnosis, staging and prognosis, monitoring response to treatment, monitoring minimal residual disease and relapse and identifying acquired drug resistance mechanisms. However, there are several obstacles to the development of cfDNA-based biomarkers which are discussed further in this in-depth review.

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INTRODUCTION

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy in the western world. In 2012 there were 152000 and 4300 deaths from ovarian cancer worldwide and in the United Kingdom, respectively[1]. This equates to twelve women dying from ovarian cancer within the United Kingdom every day.

The majority of women presenting with EOC are asymptomatic. In those women that do experience symptoms they are often vague and non-specific. The nature of the symptoms means that women generally present to their doctor with advanced stage disease. This has led to ovarian cancer being termed the "silent killer". There have been major publicity campaigns, both regionally and nationally, involving the Department of Health and cancer charities to increase the level of awareness of ovarian cancer symptomatology; in an effort to improve earlier diagnosis.

EOC - FIVE DISTINCT DISEASES

There are five main types of EOC and these are included in the current World health Organization (WHO) classification: High-grade serous carcinoma (HGSC, 70%), endometrioid carcinoma (EC, 10%), clear cell carcinoma (CCC, 10%), mucinous carcinomas (MC, 3%), and low-grade serous carcinomas (LGSC, 3%)[2]. HGSC accounts for approximately 70% of all EOCs and approximately 90% of advanced stage EOCs (FIGO stage III-IV), making it the most common and most deadly.

The EOC types differ significantly morphologically, clinically, and at a molecular level. LGSC exhibit *KRAS, BRAF,* and *ERBB2* mutations in around two thirds of cases whereas *TP53* mutations are very rare in these tumours[3,4]. *CTNNB1* (encoding β-catenin) and *PTEN* mutations, along with microsatellite instability, are associated with low-grade ECs *via* specific signalling pathways[5,6]. MC display *KRAS* mutations in more than 50% of specimens and identical mutations have been elucidated in benign, borderline and malignant areas from the same neoplastic lesion suggesting that a *KRAS* mutation is an early event in mucinous tumour pathogenesis[7]. EC and CCC have been shown to be associated with endometriosis in the ovary or pelvis in 15% to 70% of cases; this is almost certainly an underestimate since endometriosis may be overgrown by the tumour and it is likely that a large majority of EC and CCC arise in endometriosis[8,9]. In fact, there is recent evidence to show that long interspersed element-1 hypomethylation is an early molecular event involved in the transformation of EC and CCC from endometriosis[10]. The tumour suppressor gene, AT-Rich Interaction Domain 1A (*ARID1A*) also seems to play a role in early malignant transformation of endometriosis to EC or CCC[11-13]. Mutations in this gene have been identified in up to 50% of CCCs and approximately one third of ECs[12].

HGSC differs significantly from the other subtypes. The classical histological appearance is of intermediate sized tumour cells, marked nuclear atypia, and necrotic areas (Figure 1). Immunostaining with WT1, PAX8, P16, and P53 assist with the diagnosis. Interestingly, WT1 staining helps discriminate between HGSC and pseudo-endometrioid. Molecularly, it is characterised by the ubiquitous presence of *TP53* mutations and *CCNE1* gene (encoding cyclin E1) amplification in 20% of cases[14-19]. It is, however, rarely associated with mutations such as *KRAS*, *BRAF*, *ERBB2*, *HER2*, *PTEN*, *CTNNB1*, *ARID1A* and *PIK3CA*[6,7,14,15,16,19]. Germline mutations in the *BRCA1* or

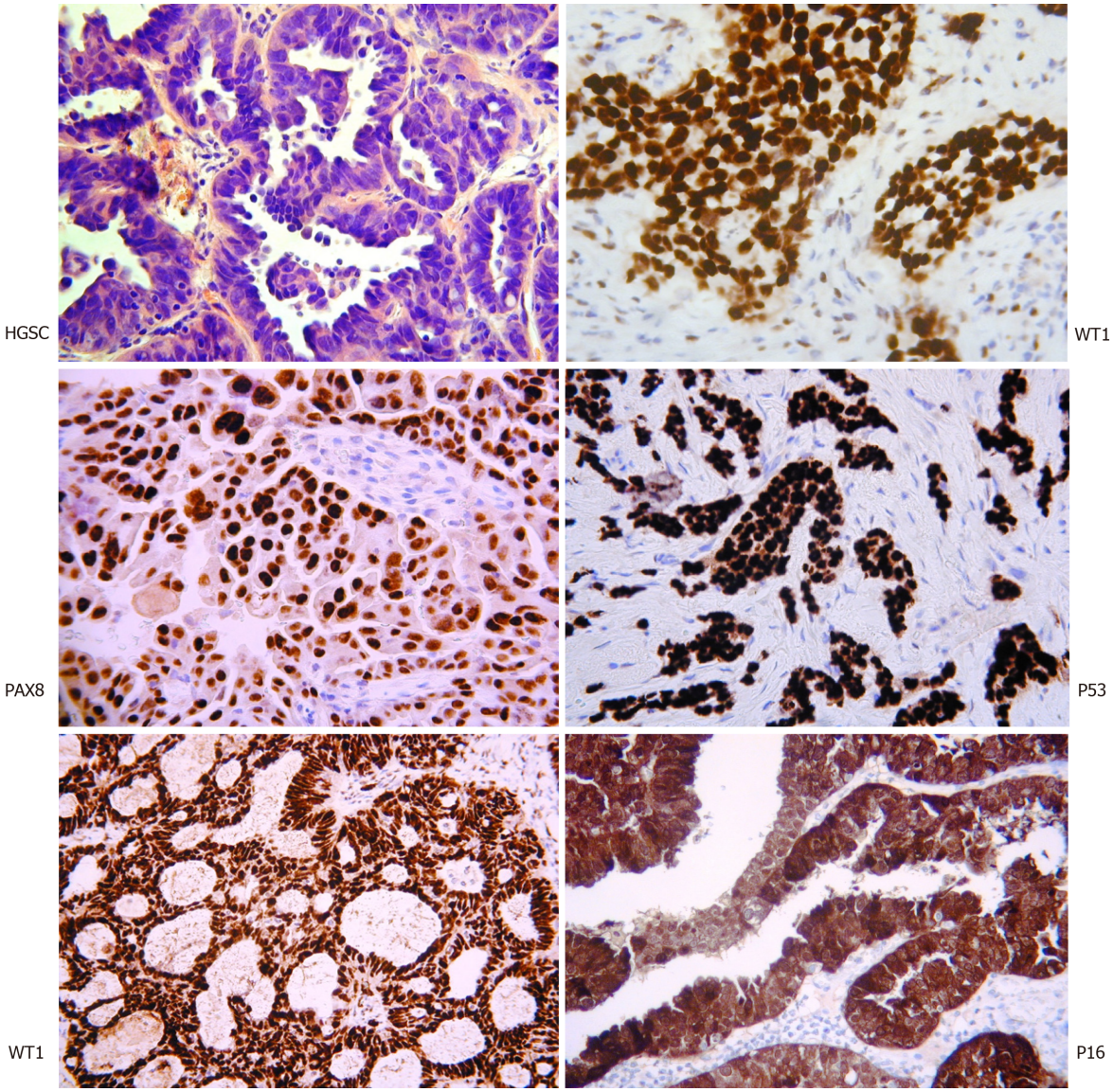

Figure 1 Histopathological assessment of high-grade serous carcinoma. The classical appearance on hematoxylin and eosin with intermediate sized tumor cells, marked nuclear atypia, and necrotic areas. Immunostaining with WT1, PAX8, P16, and P53 assist with the diagnosis. Interestingly WT1 staining helps discriminate between high-grade serous carcinoma and pseudo-endometrioid (bottom left) (Original figure, images courtesy of Professor McCluggage WG).

BRCA2 genes are present in 6.5%-19% of HGSCs and a smaller proportion have somatic mutations[20]. Whilst *BRCA1* somatic mutations are rare in sporadic disease (< 10%), BRCA1 is reported to be downregulated in 15%-72% *via* mechanisms of epigenetic inactivation[20]. To complicate things further, the HGSC subtype is also highly molecularly heterogeneous, with the possible inclusion of further subpopulations that display distinct gene expression profiles and variable responses to current chemotherapy regimens[21].

BLOOD-BASED DIAGNOSTICS OF EOC

The diagnosis of women with symptoms suspicious of EOC utilises the biomarker serum cancer antigen 125 (CA125), which was first described by Bast *et al*[22] in 1981. It was identified by the murine monoclonal antibody OC-125 as an antigenic determinant on a high molecular-weight glycoprotein. In adults, CA125 is expressed in tissues derived from both coelomic and Mullerian epithelia. It is also expressed by epithelia of the pancreas, colon, gall bladder, lung, kidney, and stomach[23].

In clinical practice, serum CA125 and preliminary radiological imaging, in the form of an abdomino-pelvic ultrasound, results are used to calculate the risk of malignancy index as a method of triaging patients for tertiary/quaternary referral[24]. Although it is

used to aid diagnosis, only 50% of early stage EOCs show expression[23,25].

CA125 is most effective as a marker of disease status in patients undergoing chemotherapy treatment for EOC[26,27]. Unfortunately, it is not specific to malignancy, expressed by several other benign conditions including diverticulitis, endometriosis, liver cirrhosis, uterine fibroids, menstruation, pregnancy, pelvic infection, and uterine leiomyomata[28-33]. It is also elevated by other malignancies such as pancreatic, bladder, breast, liver, and lung cancers[33].

With earlier diagnosis the key to improved survival from EOC, there has been considerable effort to identify alternative biomarkers or develop combination markers with CA125, without overwhelming success[34]. To improve the sensitivity of CA125 the Risk of Ovarian Cancer Algorithm (ROCA) was developed[35]. This algorithm compares the CA125 level of cases with a profile of healthy women. It calculates a risk estimate based on this comparison and the age-specific incidence of EOC. The algorithm was employed within the UKCTOCS trial and various others with varying degrees of success[36-38].

The only novel biomarker that has showed promise, since CA125, is human epididymis protein 4 (HE4)[39]. This biomarker has been shown to improve diagnostic accuracy, both alone and in combination with CA125[29,40-42]. A recent meta-analysis looked at diagnostic performance with two control groups (healthy women and women with benign gynaecological disease)[40]. In the analysis *vs* "healthy women" the sensitivity and specificity for HE4 in diagnosing ovarian cancer were 83% (95%CI: 77%-88%) and 90% (95%CI: 87%-92%), respectively. Receiver operator characteristic (ROC) analysis revealed an area under the curve (AUC) of 0.9271. In the women with benign disease analysis, the sensitivity and specificity were 74% (95%CI: 69%-78%) and 90% (95%CI: 87%-92%) respectively. The ROC analysis AUC was 0.8853. This suggests HE4 carries potential as an early-warning biomarker. Contrastingly, another meta-analysis looking at CA125 and HE4 combined diagnostic performance showed HE4 to be no better than CA125 for predicting EOC[43].

A panel of biomarkers that included CA125, HE4, transthyretin, CA15.3, and CA72.4 was evaluated using specimens assembled from multiple cohort and randomised trial[44]. Phase II and III biomarker studies concluded that CA125 remained the "single-best biomarker" for EOC. Another retrospective study evaluated seven proteomic biomarkers (apolipoprotein A1, truncated transthyretin, transferrin, hepcidin, beta-2 microglobulin, connective tissue activating protein III, and inter-alpha-trypsin inhibitor heavy-chain) in pre-diagnostic blood samples[45]. The addition of the seven protein biomarkers to CA125 did not improve sensitivity compared to CA125 alone.

The combination of CA125 and HE4 was developed into an algorithm in an effort to improve EOC detection[46]. The Risk of Ovarian Malignancy Algorithm (ROMA) successfully classified 93.8% of EOC patients as high risk. The model has been assessed in a range of populations with varying degrees of accuracy[42,47-53]. It is for this reason that HE4 or ROMA has not translated into routine clinical care, with some groups requesting further in-depth validation.

CATCHING THE SILENT KILLER: ATTEMPTS TO DATE

The evidence that population screening impacts cancer-specific mortality is ever-accumulating. There are examples of successful cancer screening programmes across many countries, particularly for breast, bowel, and cervical cancers. A significant reduction in mortality has been seen in the latter[54]. The real success of these screening programmes is the understanding of the pathogenesis of the disease. Currently there is no effective screening method for ovarian cancer and the United States Preventative Services Task Force (USPSTF) maintains its position that it should not be undertaken, in any form[55].

Ovarian cancer is relatively rare with a low prevalence in the general population. An effective and acceptable screening strategy must have not only a high sensitivity for early-stage disease (> 75%) but must also have a very high specificity (99.6%) to achieve a positive predictive value (PPV) of 10%. In real terms, that equates to a threshold of no more than 10 staging laparotomies to identify one ovarian cancer[56].

The main methods of ovarian cancer screening assessed to date are pelvic ultrasound scanning and serially measuring the serum biomarker, CA125. Pelvic ultrasound in expert hands is a highly sensitive diagnostic method[57]. Unfortunately, because it relies heavily on individual expertise, discrimination between benign and malignant pelvic masses in routine clinical practice is challenging. Serum CA125 is

most effective as a marker of disease status in patients undergoing chemotherapy treatment for EOC[26]. It is not particularly specific to malignancy and can be expressed by a number of other benign conditions including endometriosis, pelvic infection, and uterine leiomyomata. It is elevated in only 50% of early stage EOCs, and thus can cause unnecessary medical intervention and significant patient distress[25].

There have been a few large prospective trials examining ovarian cancer screening. Each utilised different screening strategies but all aimed to assess the same primary outcome: Mortality.

The United States Prostate Lung Colorectal Ovarian Cancer Screening Trial (USPLCO) comprised over 78000 women aged 55-74 years in two arms; annual transvaginal ultrasound and serum CA125 *vs* routine clinical care[58]. The study showed no difference in mortality between the two cohorts. The United Kingdom Collaborative Trial in Ovarian Cancer Screening (UKCTOCS) is the largest screening trial to date, enrolling over 200000 postmenopausal women randomised into no intervention or quarterly (initially annual) screening[38]. The screening cohort was broken down into transvaginal ultrasound alone or serum CA125 screening, interpreted by the ROCA algorithm, with second-line transvaginal ultrasound (termed multimodal screening). UKCTOCS finally reported mortality data in 2016. At a median follow-up of 11.1 years, ovarian cancer was diagnosed in 1282 (0.6%) women. There was no difference across subgroups with 0.7% in the multimodal screening group, 0.6% in the ultrasound only group, and 0.6% in the control group. Of these women, 0.29% in the MMS group, 0.30% in the USS group, and 0.34% in the control group died of ovarian cancer. The primary analysis revealed a non-statistically significant mortality reduction of 15% ($P = 0.10$) with MMS and 11% ($P = 0.21$) with USS alone.

Although the primary end point did not reach statistical significance, further analysis suggested improved mortality reductions for the MMS group compared to the control group after 7 years; 8% reduction for the first 7 years compared to 23% for years 7 to 14; suggesting a "late effect" mortality benefit[38]. In response to the "late effect" mortality trend proposed by the UKCTOCS study, extended mortality results were reported for the USPLCO trial with a median 15 year follow up (range 13 to 19 years)[59]. However, following this extended analysis, the study reiterated its initial findings and reported no change in the mortality benefit of screening using CA125 and TVUS.

NEW PATHOLOGICAL KNOWLEDGE

To date there have been no disease-specific biomarkers validated for any of the five main EOC types. Moving forward, this approach would be more appropriate given that these represent different tumour types with a different pathogenesis and behaviour and require different management. Currently, the spotlight is on HGSC as it is the most frequent and most aggressive type of EOC.

For some time, scientists and clinicians have been unable to identify a pre-invasive stage to HGSC, and the disease did not appear to fit this model. This is likely explained by the fact the disease spreads so aggressively quite early in its course, making the pathological detection of early stage disease elusive[60]. There has been considerable effort employed to define the molecular mechanisms of HGSC and, until recently, its pathogenesis remained undefined. Historically, it was thought HGSC developed from the ovarian surface epithelium (OSE) due to errors in cell replication associated with the repair of trauma incurred by ovulation[61]. Several epidemiological studies supported this theory with evidence that women with an increased number of lifetime ovulations are at a much greater risk of developing HGSC[62-67].

However, in recent years, overwhelming pathological evidence has emerged that supports the theory that the distal fallopian tube (fimbria) is the origin of HGSC[68]. The fact that fallopian tube epithelium is embryologically mullerian and HGSCs are mullerian in nature suggests a potential site of origin here for HGSC. Furthermore, malignant transformation of fallopian tubal epithelium yields almost exclusively HGSC[69]. HGSC occurring concurrently with fallopian tubal mucosal disease was first documented by Bannatyne *et al*[70] but this was interpreted to represent a second primary tumour focus rather than being directly related to the ovarian disease. Subsequently a number of research groups acknowledged that there was an increased risk of primary fallopian tubal HGSC in *BRCA1/2* mutation carriers[71-73]. Zweemer *et al*[74] presented the first evidence of primary tubal HGSC in *BRCA1* mutants and, subsequently, a large prospective study of *BRCA1* mutation carriers revealed a 120-fold increased risk, compared to the general population, of primary fallopian tube

carcinoma[75]. In 2006, Finch *et al*[76] published clinical and pathological findings of prophylactic salpingo–oophorectomy specimens from 159 *BRCA1/2* mutation carriers. Seven (4.4%) occult fallopian tube cancers were identified in these women, in the absence of symptoms. Multiple other investigators also recorded the presence of occult tubal tumours at prophylactic salpingoophorectomy[76-83]. The incidence of these occult lesions ranged from 6%-40% with up to 100% of them occurring in the fallopian tube, usually in the absence of ovarian involvement. Further investigation has revealed most HGSCs arise from the distal fallopian tube *via* an in-situ carcinomatous lesion referred to as serous tubal intraepithelial carcinoma (STIC)[84,85].

THE FALLOPIAN TUBE AS THE ORIGIN OF HGSC

There is now compelling evidence from both our group and others worldwide that most, but not all, HSGCs arise from the distal fallopian tube, and not the ovary, from STIC[86-88]. Detailed assessment of the FTs in cases of extrauterine HGSC shows involvement of the fimbriae in 70% and STIC in approximately 50% of the cases[89]. At a molecular level; Cyclin E1 (CCNE1), remodelling and spacing factor 1 (Rsf-1), and fatty acid synthase (FASN) are all upregulated in HGSC. Rsf-1 encodes an important ATP-dependent chromatin remodelling protein which is an integral component of DNA replication and cell cycle progression[90,91]. FASN is a cytoplasmic enzyme involved in tumour initiation and progression[92,93]. These oncogenes are all overexpressed in STIC lesions[94,95]. Another oncogene integral to this disease process is *TP53*[96]. Mutation of this tumour suppressor is known to be associated with HGSC and recent evidence shows it be characteristic of HGSC and essentially ubiquitous[15,97,98]. The fact that STIC lesions and concurrent HGSC have been shown to contain identical *TP53* mutations further indicates a clonal relationship between the two entities[98,99]. Gene expression profiling of a unique sample set containing normal OSE, normal FT, STIC, ovarian HGSC, and omental metastases was performed by our group. Bioinformatic analysis revealed that the tumour samples clustered in one cohort and normal FT samples clustered together and separately from the normal OSE samples. Notably, the normal OSE samples clustered separately from all other profiled samples and STIC samples clustered within the tumour cohort. Multi-dimensional scaling analysis confirmed the strong common biology present between STIC and the two tumour groups. It also affirmed that OSE has no significant common genetic biology to the other samples. This study further confirms the fallopian tubal origin of HGSC[86].

The proportion of HGSCs derived from the FT is currently unknown, mainly due to the fact HGSC usually presents at an advanced stage making the detection of precursor lesions difficult and depends on the criteria used to determine a tubal primary. However, in the most detailed prospective study, using criteria for site assignment of extrauterine HGSC adopted by the International Collaboration on Cancer Reporting (ICCR), 83% of cases were determined to be of tubal origin and almost all the remainder of ovarian origin with a primary peritoneal origin being extremely rare when strict criteria are used[100-103].

EXPLOITING THIS NOVEL MOLECULAR KNOWLEDGE: THE LIQUID BIOPSY

Precision oncology seeks to obtain molecular information about cancer to improve patient outcomes. Tissue biopsy samples are widely used to characterise tumours; however, this method of tumour analysis has limitations. The term "liquid biopsy" was first used to describe methods that can derive the same diagnostic information from a blood sample, or other body fluid, that is typically derived from a tissue biopsy sample[104]. In recent years, the focus of precision medicine is increasingly turning towards liquid biopsies as they are minimally invasive and can be repeated at multiple time points facilitating "real-time" disease monitoring[105].

Liquid biopsy (Figure 2) can include measurement of soluble factors, such as circulating tumour nucleic acids (DNA/RNA), circulating tumour cells (CTCs), proteins, and extracellular vesicles such as exosomes. All of which have been investigated for potential as diagnostic, predictive and prognostic biomarkers[106].

CTCs are cells originating from a solid tumour that are detectable in the peripheral blood. They are considered a prerequisite step in establishing distant metastases[107]. The detection of CTCs in peripheral blood (Figure 3) is a novel type of cancer

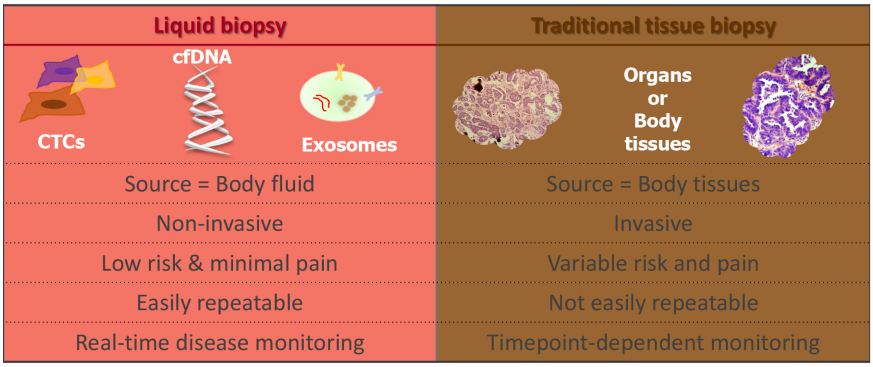

Figure 2 The difference between liquid and traditional tissue biopsy (Original figure).

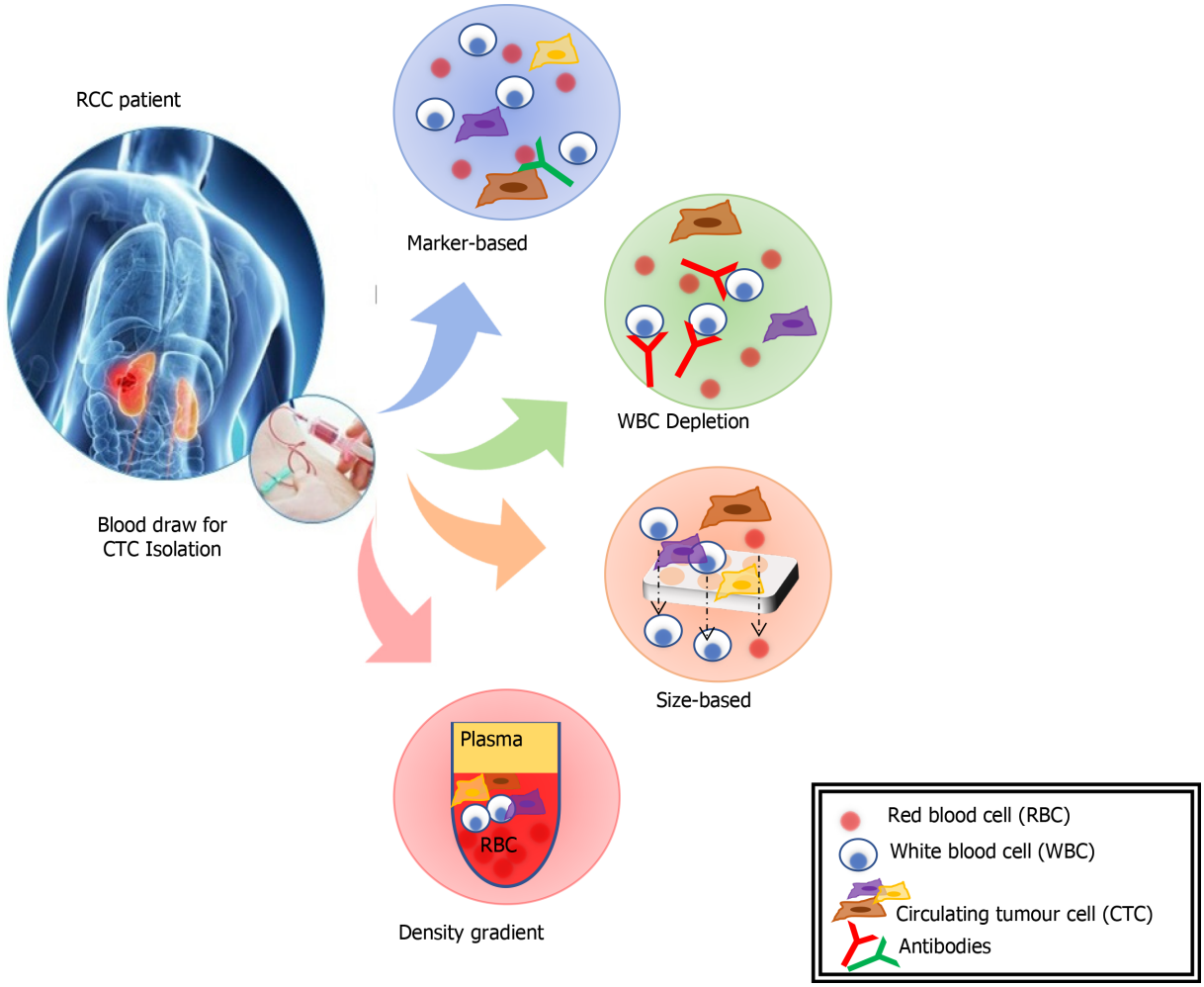

Figure 3 The extraction of circulating tumor cells can be undertaken by a range of methods (reproduced from Broncy *et al*[166] 2018 under CC BY-NC 4.0). RCC: Renal cell carcinoma.

biomarker[108]. CTCs can be isolated from blood samples and used to follow patients over time. They can provide significant information that will better characterise underlying disease biology and metastases. However, CTCs are rare, and their isolation, quantification and molecular characterization carry many challenges. An average metastatic carcinoma patient has between 5 and 50 CTCs for every 7.5 mL of blood[109]. This places technical limitations on the ability to identify and characterise the sub-population of cells that carry the relevant genetic information. CTCs tend to aggregate with leucocytes so adequate cell-surface markers and separation techniques need to be utilised to improve purity[109].

CTCs were first detected on the background of malignant melanoma and have

subsequently been identified associated with a range of solid tumours[110]. There are a range of approaches for isolation of CTCs including; mRNA-based, protein-based, and cell size-based. The mRNA-based strategy involves RqPCR techniques. This carries some limitations including: (1) Amplification of non-specific products, (2) Lack of validated protocols for sample processing, RNA-preparation, cDNA synthesis and PCR conditions, and (3) A lack of sample quality control measures[107]. These issues all raise the possibility of variations in sensitivity and specificity of a biomarker employing this method. Separation and enrichment of CTCs, using magnetic bead technologies, followed by flow cytometry or immunohistochemistry is another method of quantification and characterisation of CTCs[106]. This method eliminates some of the concerns with RqPCR techniques, but further research needs to be done to clarify the reproducibility of different techniques. The isolation by size of epithelial tumour cells is a direct method for CTC identification and has been applied in various epithelial cancers[107]. The CTCs are collected by filtration and, following staining for specific markers, the cells are identified and quantified by immunohistochemistry or molecular pathological techniques.

The only clinically validated, FDA-approved blood test for CTCs is the CELLSEARCH® CTC Test (Janssen Diagnostics, New Jersey, United States). It has shown promise as a prognostic indicator in prostate, colorectal, and breast cancers[111-113]. This technology was assessed within the EOC population and while it did provide evidence that ovarian CTCs were present in blood it did not correlate with clinical outcomes[113]. A further study assessed a unique cell adhesion matrix based, functional cell enrichment and identification platform[114]. There was clear evidence that elevated CTCs correlated with worse OS and PFS. In fact, this method proved more specific than CA125 in detecting EOC malignancy in high-risk patients. A recent meta-analysis assessed eleven studies comprising a total of 1129 patients[115]. It reported CTC status to be a significant prognostic indicator (OS:HR 1.61, 95%CI: 1.22-2.13; PFS:HR 1.44, 95%CI: 1.18-1.75). A subgroup analysis showed the RqPCR methodology to be superior to both CELLSEARCH® and immunohistochemical methods.

Despite the potential role of CTCs in cancer diagnostics, CTC methods are generally used for research purposes, and only a few methods have been accepted for clinical application. This is because of the difficulties caused by CTC heterogeneity, CTC separation from blood, and a lack of thorough clinical validation[116].

In recent years there has been a significant amount of research into the use of circulating cell free DNA (cfDNA) as a biomarker. The presence of circulatory cfDNA was identified over seventy years ago[117]. Along with technological evolution, subsequent research has demonstrated that cancer cells release cfDNA fragments into the circulation and other bodily fluids, termed circulating tumour DNA (ctDNA), and these fragments carry all the genetic and epigenetic characteristics of the primary tumour[118]. Fragments of cfDNA in blood samples are between 150 and 200 bp long, of which up to 90% originates from the tumour[118]. It is reported that tumours containing approximately 50 million malignant cells release enough DNA for the detection of tumour cfDNA in blood[119]. Of note, this is well below the limit of resolution of radiological imaging (approximately 1 billion cells)[120].

As a result, ctDNA analysis has emerged as a potential blood-based "liquid biopsy" for early detection, diagnosis, staging and prognosis, monitoring response to treatment, monitoring minimal residual disease and relapse and identifying acquired drug resistance mechanisms.

There are several hypotheses as to the origin and mechanism of release of cfDNA/ctDNA into the circulation; however, the precise mechanism(s) have yet to be determined. Early studies suggested that ctDNA enters the circulation following lysis of cells on the interface between the tumour and circulation[121]. Another theory proposed that ctDNA may originate from the destruction of tumour micro metastases and circulating cancer cells[122]. Current consensus suggests that most cfDNA in healthy individuals is released from the bone marrow and white blood cells, whereas ctDNA in cancer patients is derived from necrotic and apoptotic cancer cells. Three possible mechanisms resulting in the shedding of DNA from both healthy and tumour cells have been described: Apoptosis, necrosis, and active cellular release[123]. Apoptosis causes the systematic cleavage of chromosomal DNA into multiples of 160-180 bp stretches, resulting in the extracellular presence of mono-(approximately 166 bp) and poly-nucleosomes (332 bp, 498 bp)[124]. Necrosis results in nuclear chromatin clumping and non-specific digestion, producing DNA fragments that are typically larger than 10000 bp. DNA fragments derived from active cellular secretions have been shown to range between 1000 and 3000 bp. Most of the DNA present in plasma occurs as fragments around 180 bases and 360 bases in size and reflects the likely apoptotic

origin of the DNA[125].

In cancer patients, cfDNA may originate from multiple sources, including cancer cells, cells from the tumour microenvironment and normal cells such as haematopoietic stem cells, muscle cells and epithelial cells. Once present in circulation, cfDNA levels are influenced by multiple factors including its: (1) Dynamic association and disassociation with extracellular vesicles and several serum proteins, (2) Rate of binding, dissociation and internalisation by cells; and (3) Rate of digestion or clearance, including the activity of deoxyribonuclease I (DNAse), renal excretion into urine, and uptake by the liver and spleen[126]. Furthermore, cfDNA levels can be elevated due to the lysis of white blood cells (WBCs) and release of germline DNA, thus diluting ctDNA concentrations[127]. For this reason, it is crucial that during molecular analysis tumour-specific ctDNA is differentiated from non-tumour cfDNA and contamination of blood samples through WBC lysis is kept to a minimum.

THE LIQUID BIOPSY IN CLINICAL PRACTICE

Disease staging

Studies have demonstrated that cfDNA levels in cancer patients are generally higher than those of healthy subjects. cfDNA is present in healthy individuals at average concentrations of 30 ng/mL, ranging from 0-100 ng/mL[118]. In cancer patients, given the additional release of cfDNA from tumour cells, the average concentration of cfDNA is much higher, at approximately 180 ng/mL[128]. cfDNA concentration has also been shown to correlate with tumour size, disease stage and metastatic burden[129-131].

In a study of 640 patients with different cancer types at varying stages, Bettegowda *et al*[131] showed that cfDNA levels were approximately 100 times higher in stage IV disease compared to stage I disease, providing a rough estimate of tumour size based on cfDNA concentration. In another study, aimed at providing a more sensitive metric for estimating tumour size, researchers reported that mutant alleles increased by 6 mutant copies per ml of plasma for every cubic centimetre of tumour in participants with HGSC[129].

In one of the first studies to examine cfDNA concentration in EOC, Kamat *et al*[130] demonstrated that cfDNA levels were elevated in advanced EOC compared to normal controls. In a more recent study, the same group evaluated the role of preoperative total plasma cfDNA levels in predicting clinical outcomes in patients with EOC[132]. Again, they reported significantly higher cfDNA levels in the EOC group (median 10113 genomic equivalent/mL, GE/mL) compared with benign ovarian tumours (median, 2365 GE/mL; $P < 0.001$) and unaffected controls (median, 1912 GE/mL; $P < 0.001$). Moreover, a statistically significant association of cfDNA > 22000 GE/mL with decreased PFS ($P < 0.001$) was observed, which was superior to CA125 in predicting mortality. Furthermore, the study reported elevated cfDNA levels in patients with early stage disease which was significantly higher compared with those with benign disease and controls ($P < 0.01$). Shao *et al*[133] also reported significantly elevated cfDNA levels in advanced stage OC compared to early stage ($P < 0.01$). In contrast, No *et al*[134] reported no significant difference between cfDNA levels in EOC patients compared to controls. In this study, preoperative blood samples of 36 EOC patients and 16 benign tumours were analysed using commercially available copy number assay kits to measure cfDNA levels of four genes; beta-2-microglobulin (*B2M*), member RAS oncogene family (*RAB25*), claudin 4 (*CLDN4*) and ATP-binding cassette subfamily F member 2 (*ABCF2*). This result may be explained, in part, by the fact that, unlike the previous studies, this study used serum instead of plasma as the cfDNA source.

In a study investigating the presence of tumour-specific *TP53* sequences in blood and peritoneal fluid in EOC patients, Swisher *et al*[135] detected 30% (21/69) of patients with confirmed *TP53* mutations (exon 2-11) in plasma or serum samples. cfDNA was detected in 93% (28/30) of cases in peritoneal fluid, including six cases with negative cytology. Following multivariate analysis, they concluded that detection of cfDNA was associated with decreased survival ($P = 0.02$). Dobrzycka *et al*[136] investigated the prognostic significance of cfDNA and blood plasma p53 antibodies (p53-Ab) in EOC. Serum p53-Ab is predominantly associated with *TP53* gene missense mutations and *TP53* accumulation in the tumour. cfDNA and p53-Ab were more frequently detected in patients with HGSC ($P < 0.001$) compared to other EOC subtypes. Prognosis was significantly worse in cfDNA positive patients compared to cfDNA negative ($P = 0.022$). Similarly, patients who were p53-Ab positive had a significantly worse prognosis than those who were p53-Ab negative ($P < 0.001$).

Whole exome sequencing (WES) and targeted gene sequencing has been employed

to identify tumour-specific mutations in gynaecological cancers[137]. Subsequent monitoring of these mutations using ctDNA and digital PCR technology, in patients with gynaecological cancers, detected the recurrence of cancer, on average, seven months before disease was visible on cross-sectional imaging. Furthermore, undetectable levels of ctDNA at six months following initial treatment was associated with significantly improved progression free and overall survival.

The correlation between ctDNA levels and tumour stage/size highlights the potential prognostic value of ctDNA. Several studies have demonstrated an association between ctDNA and survival outcome in a range of cancers[138].

Personalised therapy

Assessing the mutational profile of cancer patients is used as a stratification tool to identify those who may be suitable for targeted therapies. For example, in patients with non-small cell lung cancer (NSCLC), the tyrosine kinase inhibitors (TKI) gefitinib and erlotinib are only beneficial to those with an activating mutation (L858R or exon 19 deletion) in the epidermal growth factor receptor (*EGFR)* gene[139]. Similarly, patients with malignant melanoma will only benefit from *BRAF* therapy if they harbour an activating *BRAF* mutation (V600E)[140]. Identifying these mutations and others enables clinicians to alter therapies accordingly and optimise patient care.

Historically, the assessment of mutational status has been carried out using tissue biopsies, however, this practice has some inherent disadvantages. The analysis of a single tissue biopsy taken from a primary tumour or metastatic site is likely to underestimate the mutational landscape of a tumour and can lead to inaccurate classification. Performing several biopsies on one patient can be impractical and extremely invasive. Furthermore, tissue biopsies are associated with complications, such as infection and pain, and often fail to obtain enough material for high quality mutational profiling. Numerous studies, in various malignancies, have shown that these limitations may be overcome by mutational profiling of cfDNA[106].

In CRC, high concordance between tissue and plasma samples have been reported for the detection of *KRAS, NRAS* and *BRAF* mutations, with concordance rates of 91.8% reported in one study for *RAS* mutations[141]. Another study suggested that cfDNA could replace tumour-tissue analysis resulting in considerable reductions in data turnaround time[142].

There are a limited number of studies investigating the role of cfDNA and treatment resistance in EOC. Murtaza *et al*[143] carried out WES in serial plasma samples to track the genomic evolution of metastatic cancers in response to treatment. Three patients with advanced EOC were included in the study. Quantification of allele fractions in plasma identified mutant alleles association with emerging treatment resistance. This study established a proof-of-principle that WES of ctDNA could complement current invasive biopsy approaches to identify mutations associated with acquired resistant in advanced cancers.

Overall, these studies demonstrate high concordance between tissue biopsies and plasma samples with higher diagnostic accuracy recorded using cfDNA analysis in most cases.

Treatment monitoring

The short half-life of cfDNA, coupled with the minimally invasive nature of venepuncture compared to tissue biopsies makes cfDNA an attractive tool for monitoring treatment response and disease burden (Figure 4). Numerous studies have demonstrated that low levels of cfDNA are associated with a positive treatment response in a range of cancers, including EOC[144]. In contrast, high levels of cfDNA generally correlate with poor response to treatment, treatment resistance, high risk of relapse and poor survival. In the majority of these studies, cfDNA was reported to monitor response to treatment more accurately than traditional methods. In one example, Capizzi *et al*[145] investigated the role of cfDNA levels in predicting response to chemotherapy in EOC. In this prospective non-randomised clinical trial, 22 patients with advanced EOC (FIGO stage IIIC or IV) undergoing neo-adjuvant chemotherapy were recruited alongside 50 female healthy blood donor controls. Plasma cfDNA levels were quantified before, during and after chemotherapy. Median cfDNA levels were reported to be significantly higher in the cancer group (29.6 ± 22.7 ng/mL) prior to chemotherapy, compared to controls (6.4 ± 4.0 ng/mL), with a sensitivity of 77% and specificity of 96%. A general trend was found between elevated cfDNA levels on completion of chemotherapy and disease progression (P = 0.007), however, the sample size was too small to provide conclusive survival data.

In a retrospective study of 40 patients with relapsed HGSC, Parkinson *et al*[129] used sequence specific assays to detect predefined *TP53* mutations and quantified the *TP53*

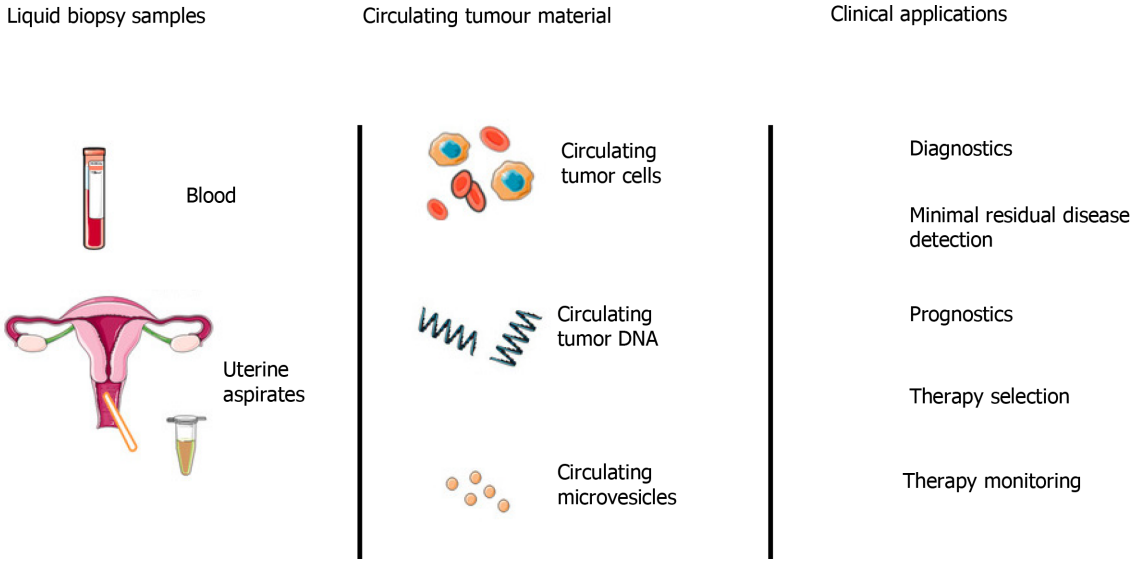

Figure 4 Potential liquid biopsy sources (blood, uterine/cervical aspirates) and downstream clinical applications within epithelial ovarian cancer (reproduced from Muinelo-Romay *et al*[167] 2018 under CC BY-NC 4.0).

mutant allele frequency (*TP53*MAF) in cfDNA using digital PCR before, during and after chemotherapy. Pre-treatment ctDNA *TP53*MAF concentration was positively correlated with total volume of disease, and a decrease of > 60% after one cycle of chemotherapy was associated with longer PFS.

Identifying treatment-resistant disease

Acquired drug resistance may emerge as a result of *de novo* mutations or the expansion of a sub-clonal cell population with pre-existing resistance[146]. The underlying mechanisms of acquired resistance are poorly understood. However, longitudinal sampling and analysis of cfDNA can provide valuable insight into the molecular response of cancer during treatment.

cfDNA can be used to monitor the development of resistance by screening for known mutations associated with resistance. Using a digital PCR assay, Ishii *et al*[147] examined cfDNA in plasma from patients with relapsed NSCLC to identify resistance mutations, namely T790M mutations, associated with EGFR-TKIs. T760M mutation was detected in plasma with a sensitivity of 81.8% and specificity of 85.7%, and overall concordance between plasma and tissue samples was 83.3%. This study showed that digital PCR analysis of plasma is a feasible and accurate alternative to tissue biopsy for detecting T760M mutations in NSCLC patients that become resistant to EGFR-TKIs.

Serial profiling of cfDNA can identify resistant sub-clones before the onset of clinical progression and enable earlier intervention. In a study investigating the acquired resistance to anti-EGFR treatment in CRC, 60% (6/10) patients showed the emergence of secondary *KRAS* mutations up to four months before an increase in the conventional marker (CEA) was detected, and nine months prior to radiological evidence of relapse[148]. This study also showed that although tumour cells exhibited resistance to EGFR inhibitors, they remained sensitive to a combination of EGFR and MEK inhibitors, enabling early and personalised treatment adjustment.

A key resistance mechanism to platinum-based chemotherapies and PARP inhibitors in *BRCA*-mutant cancers is the acquisition of *BRCA* reversion mutations that restore protein function. Lin *et al*[149] performed targeted next-generation sequencing of cfDNA extracted from plasma collected prior to rucaparib treatment in 112 patients with germline or somatic *BRCA*-mutant HGSC enrolled in the ARIEL2 study. They found *BRCA* reversion mutations in cfDNA from 18% (2/11) of platinum-refractory and 13% (5/38) of platinum-resistant patients, compared with 2% (1/48) of platinum-sensitive patients (P = 0.049). Furthermore, patients without *BRCA* reversion mutations detected in pre-treatment cfDNA had significantly longer rucaparib PFS than those with reversion mutations (median, 9.0 mo *vs* 18 mo; HR, 0.12; P < 0.0001).

In summary, analysis of cfDNA collected before and after treatment can provide a more comprehensive view of the genetic response of a patients' tumour, including the dynamic changes in the mutational landscape as well as the heterogeneity that develops due to the selective pressure of therapy.

Minimal residual disease

Another important aspect of cancer management is deciding whether further treatment is required following tumour resection. Decision-making for adjuvant therapy is based on disease stage and certain high-risk clinical or pathological features. As there is, currently, no effective tool of identifying patients with complete tumour resection this practice leads to potential under- and over-treatment with the associated consequences.

cfDNA analysis has shown great promise in the detection of minimal residual disease (MRD) and subsequent risk of recurrence. In one of the first studies to evaluate the use of cfDNA as a biomarker of MRD, Diehl *et al*[150] showed that tumour derived cfDNA levels decreased by 99% within 24 h after complete surgical resection in CRC, whereas in cases of incomplete resection cfDNA levels did not change significantly and in some cases increased. Furthermore, in subjects with detectable levels of cfDNA after surgery relapse generally occurred within one year. cfDNA levels appeared to be a more reliable and sensitive indicator than the conventional biomarker (carcinoembryonic antigen) in this cohort. More recently, Chaudhuri *et al*[151] demonstrated the potential of cfDNA deep sequencing analysis in predicting prognosis in patients who had completed potentially curative treatment for early-stage NSCLC with surgery or radical radiotherapy. They retrospectively analysed blood samples from a cohort of 40 patients, with plasma samples taken before treatment and every 2 to 6 mo during follow-up. ctDNA was detectable in 93% (37/40) of patients before any treatment and was detectable in 54% of patients after treatment, all of whom went on to relapse. Detection of ctDNA postoperatively had a very high risk of future relapse (HR, 43.4; 95%CI, 5.7–341), with a median 5.2-mo lead time over clinical progression. Further studies have reported similar findings in a range of malignancies, including EOC[126].

Although these studies are encouraging there remains a large proportion of patients who relapse in whom cfDNA is not detected. Furthermore, the vast majority of these cfDNA assays are patient and mutation specific, limiting their clinical application to wider patient populations. In order to overcome these limitations further research is required to establish highly sensitive cfDNA assays that enable the identification of patients likely to benefit from adjuvant treatments and avoid the adverse effects resulting from unnecessary treatments.

CATCHING THE SILENT KILLER: A BETTER WAY?

Despite years of research in this area, the diagnosis of early stage cancer remains extremely challenging. Recent research suggests that technological advances in the analysis of cfDNA may provide a solution to these challenges. Studies have shown that cancer-associated mutations can be detected in cfDNA in early-stage disease, before the presence of symptoms and up to 2 years before cancer diagnosis[152-156].

Cervical screening tests have revolutionised the management of cervical cancer by enabling early detection of preinvasive disease. Recently, the traditional Papanicolau smear has been replaced, in many countries, by a liquid-based cytology (LBC) method. Kinde *et al*[152] exploited this method of DNA collection to develop an assay to detect endometrial and ovarian cancer. Mutational profiling was carried out on 46 cancer patients (24 endometrial cancers and 22 ovarian cancers) for whom LBC specimens were available. The same mutations were detected in the corresponding LBC samples in 100% (24/24) of the endometrial cancers and 41% (9/22) of the ovarian cancers. The same group went on to develop the PapSEEK test using a similar technique to analysis a panel of 18 genes using multiplex PCR[157]. They reported sensitivity of 33% (95%CI, 27%-39%) in the 245 EOC patients tested, including 34% of patients with early stage disease. Specificity was approximately 99% with only 1.4% of 714 women without cancer testing positive. They also analysed plasma in 83 EOC patients for 16 genes of interest and found ctDNA in 43% (95%CI, 33%-55%), with none of the plasma samples from 192 healthy controls testing positive (specificity 100%). When combining LBC samples with matched plasma samples, sensitivity for OC was increased to 63% (95%CI, 51%-73%), including 54% with early stage disease. Although improvements are required before applying this test in routine practice, it highlights the potential to incorporate cfDNA analysis into routine screening tools such as the cervical screening programme.

Non-invasive prenatal testing (NIPT) identifies foetal aneuploidy by sequencing cfDNA in maternal plasma. Pre-symptomatic maternal malignancies have been incidentally detected during NIPT[155]. In a case control study of prospectively collected

preoperative HGSC plasma samples, Cohen *et al*[153] analysed 32 women with HGSC (16 early stage (FIGO I-II) and 16 advanced stage (FIGO III-IV)) and 32 benign controls. Plasma cfDNA was sequenced using a commercial NIPT platform. They detected 40.6% (13/32) HGSC cases using sub-chromosomal analysis, including 38% (6/16) of early stage cases. Although sensitivity was low and correlation with paired tumour DNA was not possible due to a lack of archived specimens, this study established the proof-of-principle that tumour DNA is detectable using NIPT.

The same group developed a blood test to detect eight common cancers through assessment of levels of circulating proteins and mutations in cfDNA[154]. The CancerSEEK® test was used to assess 1005 patients who had been diagnosed with stage I to III cancers of the ovary, liver, stomach, pancreas, oesophagus, colorectum, lung and breast. The median sensitivity of CancerSEEK® among the eight cancer types was 70% ($P < 10^{-96}$) and ranged from 98% in OC to 33% in breast cancers. At this sensitivity, the specificity was > 99%; with 7 of the 812 controls recorded as positive. Although sensitivity was reported as > 70% for stage II (73%) and stage III (78%) disease, only 43% of stage I cancers were detected. A key concern with this test is its achievable PPV. The prevalence of the eight cancers in healthy individuals > 64 years of age is approximately 1%. Assuming the CancerSEEK® test could achieve 99% sensitivity and specificity, the resulting PPV would be only 50%, which equates to 50% of positive tests being false positives. However, these results imply that combination strategies have the power to greatly improve liquid biopsy analyses.

Gormally *et al*[156] assessed the significance of plasma DNA mutations for subsequent cancer development in healthy subjects in a large longitudinal prospective study. The study included > 520000 healthy volunteers recruited from 10 European countries. Plasma specimens were tested for *TP53* and *KRAS2* mutations. Results showed that mutations in *TP53* and *KRAS2* could be detected in cfDNA of healthy subjects on average 20.8 mo (range, 1.8-44.8) and 14.3 mo (range, 2.6-24.9) before cancer diagnosis, respectively. *TP53* and *KRAS2* mutations were detected in 3% and 1%, respectively, of subjects who did not develop cancer during follow up. This is an important finding as it highlights the presence of high levels of oncogenic drivers in the plasma of healthy individuals. This has been attributed to a common aging-related phenomenon known as clonal haematopoiesis, in which haematopoietic stem cells or other early blood cell progenitors contribute to the formation of genetically distinct subpopulations of bloods cells[158]. The establishment of a clonal population may occur when a stem or progenitor cell acquires one or more somatic mutations that give it a competitive advantage over other haematopoietic cells. The incidence of clonal haematopoiesis rises with age and is an important consideration when evaluating potential blood-based tumour-specific biomarkers.

Whilst these studies demonstrate the potential of cfDNA as an early detection marker, several significant obstacles need to be overcome before the majority could be used in a clinical setting. The main obstacles to the development of cfDNA based biomarkers are: (1) Low abundance of ctDNA in the blood; and (2) High levels of background non-cancerous cfDNA, mostly shed from WBCs. Highly sensitive technologies are required to accurately detect scarcely abundant alleles within high background levels of nontarget molecules.

Due to this, cfDNA-based cancer biomarkers have yet to make it into the clinical arena. The greatest progress has been observed in obstetric medicine, where cfDNA has been successfully used for fetal Rhesus D genotyping, for the detection of paternally inherited genetic disorders from maternal blood, and revolutionised the identification of fetal aneuploidy through non-invasive pre-natal screening[159]. In the future this level of success may be seen for cancer-specific cfDNA biomarkers.

CONCLUSION

The use of liquid biopsies is a fast-emerging area of cancer diagnostics, in particular the detection of ctDNA, and multiple cancer types are known to produce quantifiable levels of ctDNA. Studies have shown ctDNA to be useful in monitoring for minimal residual disease, treatment response, chemoresistance, and tumour heterogeneity. ctDNA can be used as an early warning diagnostic tool, either through identification of known genetic aberrations (such as *TP53* mutations), or through measurement of cancer-specific DNA methylation (DNAme) of particular genomic loci. However, use of this technology for early diagnosis is very much in its infancy. In EOC it has been shown that *TP53* mutations can be identified in ctDNA, using tagged amplicon deep sequencing, in up to 65% of patients with advanced EOC[160]. Once optimised this could

be a very useful liquid biopsy for HGSC but it would be important to ascertain the *TP53* mutation rate in normal healthy controls as a baseline.

At present, sequencing technology allows for the detection of one mutant allele, *e.g.*, p53, in a background of 1000 wild-type molecules[161]. For this reason, the current focus of our research is on DNAme because it allows for the detection of specific patterns rather than single point mutations, potentially improving the performance characteristics and detection limit of such an assay. There have been numerous reports of alterations in methylation events occurring in EOC, including HGSC[162-164]. This could prove to be an extremely useful mechanism through which HGSCs might be detected earlier and more consistently.

The identification of a biomarker requires a number of phases of development which can be crudely described as; case and control selection, determination of detection limits and assay precision, validation in second/external datasets, statistical interpretation and ROC analysis. The failure to find suitable biomarkers for EOC, despite significant investment in the United Kingdom, Europe and the United States, led to an inquiry into possible causes. This inquiry recommended the use of the PRoBE (prospective-specimen collection, with retrospective-blinded evaluation) design for biomarker discovery and validation because it was felt that biomarkers discovered in clinical sample sets collected at diagnosis from symptomatic patients and controls in hospital settings are unlikely to fully represent the screening population[165]. The PRoBE design involves blinded case-control studies nested within a prospective cohort representing the target population. The specimens and matched clinical data will have been collected prior to knowledge of the outcome (*e.g.*, diagnosis of EOC). Paramount to the successful implementation of the PRoBE design is the knowledge of the accurate calibrator-control for the disease the biomarker is aimed for.

As discussed, the clinicopathological and molecular developments over the last decade has redefined EOC as essentially representing five distinct disease entities. It is now the time to consider identifying disease-specific biomarkers rather than a generic EOC tumour marker. This will likely yield more success and, ultimately, result in increased precision of the biomarker.

HGSC is the major contributor to morbidity and mortality under the EOC "umbrella". Therefore, an accurate disease-specific biomarker is urgently needed; not just for "screening" but for greater diagnostic accuracy and also for monitoring disease stability/progression during treatment and assessing residual disease post cytoreductive surgery. Our use of cutting-edge, high throughput, molecular assay technologies has helped clarify the underlying molecular profile of this disease[86]. Knowledge like this will guide the identification and validation of novel transcripts that carry the potential to act as disease-specific biomarkers of HGSC.

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Updates on "Cancer Genomics and Epigenomics"

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Abstract

The field of "Cancer Genomics and Epigenomes" has been widely investigated for their involvement in cancer to understand the basic processes of different malignancies. The aggregation of genetic and epigenetic alterations also displays a wide range of heterogeneity making it quite necessary to develop personalized treatment strategies. The complex interplay between DNA methylation and chromatin dynamics in malignant cells is one of the major epigenetic mechanisms that lead to gene activation and repression. Hence, each tumor needs to be fully characterized to satisfy the ideas of personalized treatment strategies. The present article addresses various aspects of genome characterization methods and their potential role in the field of cancer genomics and epigenomics.

Key Words: Genetics; Epigenetics; Chromatin; Cancer; Copy number variations; Transcription; Histone-modifications

Core Tip: Various genetic, as well as epigenetic alterations such as mutations, copy number variations, structural variations, and epigenetic dysregulations, are described as hallmarks of cancer. Understanding the complex interplay of genetic and epigenetic changes is exceedingly important for improving cancer care and the development of personalized medicine. Advanced genome analysis tools have enabled researchers to obtain a comprehensive picture of the cancer genome. However, the understanding of tumor genomics/epigenomics and the correct use of technology are tightly coupled processes. The present article summarizes various genome analytic methods utilized in

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INTRODUCTION

"Cancer Genomics and Epigenomes" has played a critical role in our understanding of the basic processes of different malignancies. Multistep tumorigenesis is a progression of events, which result from the dysregulation of signaling mechanisms and alterations in the processing of genetic information. Cancer is a heterogeneous genetic disease caused by alterations in the genes that control cell growth and division. The major changes involve mutations in our genetic material *i.e.* deoxyribonucleic acid (DNA) which makes our genes. The risk of developing cancer increases if genetic changes are inherited as germline changes and present in the germ cells. These changes are present in the entire cells of the progeny. Cancer-causing genetic changes can also be acquired during a person's life because of the errors in DNA repair mechanisms, which are caused by chemicals in tobacco, smoke, and radiation like ultraviolet rays. The genetic changes, which happen after conception at any time during the life of a person, are called somatic or acquired changes. The tumor cells can undergo various genetic changes like chromosomal rearrangements or genetic mutations.

Epigenetics is defined as heritable changes in gene activity that occur without alterations in the DNA sequence. These changes in gene expression are stable between cell divisions[1]. Some of these non-genetic variations are strongly controlled by two main epigenetic mechanisms, *i.e.* chemical modifications to the cytosine residues of DNA called DNA methylation and histone post-translational modifications associated with DNA. The complex interplay between DNA methylation and chromatin dynamics in malignant cells is one of the major epigenetic mechanisms that lead to gene activation and repression. These modifications play critical roles in the epigenetic inheritance of transcriptional memory. Complex patterns of epigenetic modifications can act as epigenetic markers to characterize gene expression, gene activity, and chromatin state[2,3]. Most epigenetic alterations are the global regulator of gene expression, affect the properties and behavior of the cells, and severely affect cancer progression.

The Cancer Genome Atlas, a database of comprehensive, multi-dimensional maps of the crucial genomic changes in 33 different cancers, that has been developed by a collaboration between the National Cancer Institute and National Human Genome Research Institute, comprises 2.5 petabytes data describing paired tumors and normal tissues of more than 11,000 patients. This database is freely available and is widely used by researchers (https://cancergenome.nih.gov/). The cBio Cancer Genomics Portal is a web-based (http://www.cbioportal.org/) open-access interactive platform of cancer genomics multidimensional datasets, which offer access to 5000 tumor samples from 20 various cancer studies. These efforts have lowered the walls between big genomic multifarious data and cancer researchers to translate the important information into biologic visions with applications to the clinic[4,5]. Nevertheless, the online cancer databases have recently evolved as a portal for integrative oncogenomics that stores data regarding gene information, microRNA (miRNA), protein expression profiling, copy number variations for several cancer forms, as well as protein-protein interaction information (http://www.canevolve.org/). It allows interrogating the consequences of the primary analysis, integrative analysis as well as network analysis of oncogenomics data[6]. A recent study published by Agarwal *et al*[7] created a Colorectal cancer Database, which contains the information from 2056 colorectal cancer (CRC)-related genes associated with different CRC stages, obtained from available literature with current information. This database is helping the clinicians obtain a better understanding of diagnosis as well as treatment and classification of CRCs http://lms.snu.edu.in/corecg/[7].

Next-generation sequencing (NGS)-based platforms such as the Ion Torrent PGM

sequencer, MiSeq, HiSeq, reduced representation bisulfite sequencing, chromatin immunoprecipitation sequencing, methylation sequencing, methylated DNA immunoprecipitation, RNASeq, and array-based techniques are recently in use and have greatly assisted the discovery of biomarkers for the early diagnosis, prognosis, responses to chemotherapy, and treatment strategies in various cancers. In addition, the establishment of advanced NGS methodologies for less explored domains of cancer genomics such as non-coding RNA (ncRNA), miRNA, long ncRNA, small nucleolar RNA, and circular RNA (circRNA) have also revolutionized cancer research.

The present article addresses various aspects of genome characterization methods and their potentials role in the field of cancer genomics and epigenomics.

SINGLE-CELL SEQUENCING IN CANCER

The biological functions of cells are attained from transcription and translation. However, errors in their process can give rise to many genetic issues. Differences in gene regulation, stochastic variation, or environmental perturbations, are reflected at the genomic, transcriptomic, and proteomic levels. Cellular heterogeneity among cells within the same tumor is a result of genetic changes influenced by the environment. The heterogeneous nature of tumors is the primary reason for complicated treatment outcomes since a treatment that targets one tumor cell population may not be effective against another. To understand the role of rare cells in tumor progression single-cell sequencing is an emerging technology. Single-cell sequencing is a strong tool for investigating clonal evolution and heterogeneousness in cancer. With the help of currently available technologies for single-cell sequencing, it has become quite possible to characterize the intra-tumor cellular heterogeneity, measure mutation rates, and identify rare cell types that give rise to cellular heterogeneity. The hypothesis behind the development of carcinoma is the process of clonal evolution from mutated cells. Currently available technologies to identify rare cell types *e.g.*, microfluidic-based single-cell sorting methods for stem cells, mass cytometry-based proteomic strategies, and high-throughput multiplexed quantitative polymerase chain reaction or sequencing methodologies. These methods provided new possibilities to look into the dynamical processes of cell-fate transitions[8].

Single-cell analysis in cancer genomics is highly important and new research orientation these days. Literature shows a good number of studies that have tried to explore the single-cell analysis approach to characterize cellular heterogeneity especially in the field of cancer genetics[9,10]. Bartoschek *et al*[10] studied the spatially and functionally distinct subclasses of breast cancer-associated fibroblasts by a single-cell RNA sequencing approach and found that there was an improved resolution of the widely defined cancer-associated fibroblast (CAF) population. The authors concluded that the single-cell RNA sequencing approach paves the way for the development of biomarker-related drugs directly for precision targeting of CAFs. Moreover, a recent review article by Macaulay *et al*[11] concluded that both genomic and transcriptomic heterogeneity within an organism has been considerably underestimated, and single-cell approaches now stand poised to illuminate this new layer of biological complexity during normal development and disease[11].

Exploring the field of cellular non-uniformity by single-cell sequencing approach will be very useful to impart fundamental insights into biological processes. Besides, it will also have important applications in the development of cancer-related therapies which is till now challenged by inter/intra heterogeneity.

COPY NUMBER VARIATIONS IN CANCER

Human genomes were once thought to be stable but current literature is full of publications with opposite views. An unexpectedly frequent, dynamic, and complex form of genetic diversity called copy number variations (CNVs) is an example of the dynamic nature of the genome. CNVs are a class of structurally variant regions within two human genomes. These discoveries have suddenly reversed the idea of a single diploid "reference genome." The DNA CNVs are an important constituent of genetic variation, affecting a larger fraction of the genome as compared to single nucleotide variation. CNVs are greater than 1 kb base in size and defined as gains/losses of genomic DNA, which are of microscopic or submicroscopic level, therefore, not necessarily visible by standard G-banding karyotyping. The figures from March 2009 until the date of the Database of Genomic Variants have ascertained approximately

21000 CNVs or around 6500 unique CNV loci. The CNVs are believed to cover around 10% of the human genome. The cutting-edge technologies (NGS and microarray) have greatly facilitated the study of various aspects of human genomes. The recent advancements in medical research have made it possible to characterize the widespread constitutional CNVs, which have highlighted their role in susceptibility to a wide spectrum of diseases. The role of CNVs in cancer is currently underestimated and less understood. The CNV data can be used to identify regions of the genome involved in disease phenotypes because it affects the larger fraction of the genome. The advent of high-resolution technologies has made it possible to identify genome-wide CNVs in comparatively less amount of time and are actively trying to determine the clinical impact of CNVs in patient populations. In particular, presence of CNVs leads to gene dosage variations and large-scale genome-wide investigations have demonstrated the large impact of CNVs on severe developmental disorders such as Down, Prader Willi, Angelman, and Cri du Chat syndromes, which result from gain or loss of one copy of a chromosome or chromosomal region. In cancer, copy number changes have also been involved in altered expression of tumor-suppressor as well as oncogenes. Thus, detection and mapping of copy number abnormalities provide significant information about the link of copy number aberrations with the disease phenotype, which ultimately helps the researchers in localizing critical genes.

TUMOR GENOTYPING IN CANCER

The development of targeted therapies for cancer patients greatly depends on tumor genotyping. A collaboration of pathologists and molecular biologists with clinical practitioners has shed a light on better, cost-effective, and comparatively faster methods to "genotype" malignant tumors to understand the genetic architecture of many cancers. Identifying the genetic abnormalities that drive particular tumors is leading to significant improvement in treatment-related modalities. It is evident from current data that genetic mutation based on targeted therapies work much better than conventional treatment methods. Moreover, personalized therapies can be common for two or more different cancers, irrespective of their tissue of origin.

MICROSATELLITE INSTABILITY AND CANCER

Genetic instability is a characteristic of most known malignancies, which involves a higher rate of mutations in the genome of cellular lineage. Genetic instability pathways are known to underlie mechanisms behind the development pattern of CRC. However, existing data concerning microsatellite instability (MSI) in hereditary colorectal cancer (85%) shows a well-marked distinction compared to sporadic CRC (up to 20%). It may be of particular interest that inactivated DNA mismatch repair (MMR) apparatus plays a crucial role in MSI occurrence, as loss or altered function of MMR proteins causes more error-prone DNA replication leading to the sequential accumulation of mutations.

MiRNA AND CANCER

miRNA or small ncRNA regularizes various target genes and hence engages in a variety of biological and pathological processes, like cancer. The role of miRNA in cancer has been discovered in several studies. Recent studies have identified mir-139-5p as a novel serum biomarker for CRC progression recurrence and metastasis[12]. CircRNA "ciRS-7-A" is a potential therapeutic prognostic biomarker for CRC[13]. A study from the Yang group identified some novel approaches for studying circRNA functions. The authors found that circ-Foxo3 regulates the cellular functions of tissue cells and suppresses cancer cell progression in breast cancer[14]. The long ncRNA, H19, is significantly associated with CRC patient survival[15]. H19 silencing blocks the G1-S transition, inhibits cell migration, and reduces cell proliferation[15]. Regulation of Let-7[16], RB1-E2F1, and activity of β-catenin are also crucial upstream regulators mediating H19 function. Moreover, these ultra-conserved elements (*i.e.* miRNA) signify a newly recognized class of ncRNA, which assist in regulating miRNAs by direct interaction. These ultra-conserved genes expression signatures can help in better classification of tumors, including CRCs[17].

Cancer pharmacogenomics targets the understanding of how genetic variants influence drug efficacy and toxicity in cancer patient treatments. The inter-individual genetic variation affects a drug's pharmacokinetics, pharmacodynamics, and response to treatment[18]. As every patient responds to drug treatment differently, the genotype-phenotype association can decide the drug dose. Pharmacogenomics-based databases have been developed in recent years for identifying patient target genes and small molecule candidates for cancer therapeutics[17]. A review article published on "cancer pharmacogenomics" in Nature genetics 2013 explains about various strategies and challenges including recent developments in sequencing techniques, clinical trial designs, and application of germline genetics analysis with bio-statistical genetics analysis methods that have potential for the detection of genetic variants associated with inter-individual response to the drug[19].

The list of genome/epigenome characterization techniques with their respective advantages and disadvantages is available in Table 1[20-30].

LIQUID BIOPSIES

Cells undergoing apoptosis or necrosis shed cell-free fragments of DNA into the bloodstream, which gives rise to circulating cell-free DNA. Recently, the technology has enabled to detect low levels of such a minor amount of cell-free DNA. In cancer, the load of circulating free DNA increases as the cells are increasingly undergoing cell division. The DNA shed by cancerous cells is known as circulating tumor DNA (ctDNA). ct-DNA contains the genetic information about the tumor and it has been evident from previous studies that ctDNA can also depict a wide range of clinical information like tumor genotyping, tumor staging, tumor grading, and prognosis, *etc*[31-34]. There are two major methods to study ct-DNA: One is by targeting ctDNA to find particular gene mutations/structural rearrangements in specific genome regions and the other one is the detection of *de novo* ctDNA mutations and somatic CNVs. These approaches are known as targeted and non-targeted approaches, respectively. The untargeted approach does not require any prior knowledge of molecular alteration, for instance, whole-genome sequencing. Both of these approaches have advantages and limitations. Genotyping ctDNA can prove a useful tool to detect any malignancy at the initial stage as well as the effect of ongoing treatment on patients without the involvement of invasive techniques. Table 2 lists the available CTC isolation methods[35-39].

CONCLUSION

In a nutshell, mutations, genome imbalances, and disruption of epigenetic machinery cumulatively characterize cancer. This evolutionary procedure for cancer progression requires interplay between the Cancer Genome and Epigenome. The newly formed malignant traits stably accumulate in the clonal lineage of cancer cells. Oncogenic events like mutation, copy number alteration, deletion, and insertion are particularly well recognized in the field of cancer biology. Cancer has been primarily studied based on genetics only. However, epigenetics turned out to be an alternative way of cancer that plays a significant role in acquiring stable oncogenic traits. Genetic and epigenetic mechanisms have a combined role in the formation of oncogenic traits by working in a multitude of ways as a team. Therefore, tumor characterization must include epigenomics along with genomics to get more accurate insights about the molecular pathology of the disease.

Table 1 Genome characterization technologies

Technique	Application	Advantages/remarks	Disadvantages	Ref.
GTG banding	Chromosomal abnormalities, such as translocations, genomic, autosomal trisomies, polyploidy, sex chromosome monosomy and double trisomies	Can only detect rearrangements that involve > 3 Mb of DNA; -low Sensitivity	-Limited to mitotically active cells; -Difficulties in decoding highly rearranged chromosomes	[20,21]
FISH	Gene fusions, aneuploidy, loss of a chromosomal region or a whole chromosome	Can identify genetic changes that are too small. High sensitivity and specificity	Heavily influenced by cellular phenomena and hybridization artifacts	[22,23]
aCGH	Deletions, amplifications, breakpoints, and ploidy abnormalities	Detect DNA copy changes simultaneously at multiple loci in a genome- sensitivity was estimated to be 96.7% - specificity was 99.1%	Inappropriate for the detection of mosaicism, balanced chromosomal translocations, and inversions	[24]
Sanger seq	Single nucleotide change, small indels (insertions or deletions)	Gold standard platform widely used for SNV detection; --High sensitivity and Specificity	Unable to detect mosaic alleles below a threshold of 15%–20% (Limit of detection 15%–20%); Low discovery power	[25]
NGS	Whole genome sequencing, de novo assembly sequencing, resequencing, and transcriptome sequencing at the DNA or RNA level	Higher sequencing depth enables higher sensitivity (down to 1%); More data produced with the same amount of input DNA; -High sensitivity and Specificity	Expensive method; High rate of sequencing errors	[26,27]
Bisulfite conversion	DNA methylation	Resolution at DNA level. Effective method providing information about cytosine methylation	Beginning with high quality DNA is critical, Impossible to distinguish methylated and hemimethylated cytosine	[28]
MDRE	Restrict CpG methylation analysis only to methylated regions of genome	Easy to use availability and assortment of endonucleases	DNA methylation assay is circumscribed by the use of a particular enzyme	[29]
ChIP-seq	-Analyze protein interactions with DNA; -ChIP-seq combines chromatin immunoprecipitation (ChIP) with NGS for identification of binding sites of DNA-binding proteins	Fast well studied. Compatible with array-or sequencing-based analysis, *i.e.* it is possible to perform genome-wide analysis	Relies on antibody specificity Microarray assay relies on particular probes	[30]

FISH: Fluorescence in situ hybridization; aCGH: Array comparative genomic hybridization; NGS: Next generation sequencing; MDRE: Methylation-dependent restriction enzyme; ChIP-seq: Chromatin immunoprecipitation sequencing; Sanger seq: Sanger sequencing; SNV: Single nucleotide variation.

Table 2 Circulating tumor cell isolation techniques

Technology	Platform	Methods	Ref.
Physical properties	Density gradient, physical filter, dielectric, photoacoustic microfluidic	Size, density	[35,36]
High-throughput imaging	Imaging cytometry	Scanning of cells on the slide	[36]
Leukocyte depletion	Batch cell lysis; microfluidic CTC-iChip; immunomagnetic separation	Negative depletion of leukocytes	[37]
Antibody capture	Cell search; magsweeper; microfluidic CTC-chip	Selection for tumor-specific markers	[37]
Functional characteristics	Epispot assay, invasion assay	Protein secretion, cell migration	[38]
Nanotechnology	Immunomagnetic nanobeads, nano-structures substrates in microchip	Nanomaterials able to increase interactions with CTCs and specific antibodies, and to enable their electrical conductivity	[39]

CTC: Circulating tumor cell.

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MINIREVIEWS

Deep diving in the PACIFIC: Practical issues in stage III non-small cell lung cancer to avoid shipwreck

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Abstract

After publication of the PACIFIC trial results, immune checkpoint inhibitor-based immunotherapy was included in the treatment algorithm of locally advanced non-small cell lung cancer (NSCLC). The PACIFIC trial demonstrated that 12 mo of durvalumab consolidation therapy after radical-intent platinum doublet

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chemotherapy with concomitant radiotherapy improved both progression-free survival and overall survival in patients with unresectable stage III NSCLC. This is the first treatment in decades to successfully improve survival in this clinical setting, with manageable toxicity and without deterioration in quality of life. The integration of durvalumab in the management of locally advanced NSCLC accentuates the need for multidisciplinary, coordinated decision-making among lung cancer specialists, bringing new challenges and controversies as well as important changes in clinical work routines. The aim of the present article is to review—from a practical, multidisciplinary perspective—the findings and implications of the PACIFIC trial. We evaluate the immunobiological basis of durvalumab as well as practical aspects related to programmed cell death ligand 1 determination. In addition, we comprehensively assess the efficacy and toxicity data from the PACIFIC trial and discuss the controversies and practical aspects of incorporating durvalumab into routine clinical practice. Finally, we discuss unresolved questions and future challenges. In short, the present document aims to provide clinicians with a practical guide for the application of the PACIFIC regimen in routine clinical practice.

Key Words: Durvalumab; Non-small cell lung cancer; PACIFIC; Immunotherapy; Immune checkpoint inhibitors; Anti-programmed cell death ligand 1; Consolidation therapy; Unresectable stage III lung cancer

Core Tip: Durvalumab consolidation therapy is the new standard of care in patients with unresectable stage III non-small cell lung cancer treated with concomitant chemoradiotherapy. In the PACIFIC trial, durvalumab significantly improved both progression-free survival and overall survival compared to placebo, with a manageable toxicity profile. However, several questions surrounding the clinical implementation of this therapy remain including optimal patient selection and timing of treatment initiation, programmed cell death ligand 1 determination, and the impact on clinical routines. These questions make interdisciplinary decision-making more necessary than ever. This review assesses these issues and future challenges from a multidisciplinary approach.

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INTRODUCTION

Lung cancer is the most common type of cancer worldwide, with 2.1 million new cases diagnosed annually; it is also the leading cause of cancer-related mortality, accounting for nearly one in five cancer deaths (1.8 million in 2018). Approximately 30% of patients with non-small cell lung cancer (NSCLC) are diagnosed at stage III, representing about 500000 new cases per year[1]. While NSCLC is potentially curable in patients with locally advanced disease, 5-year survival rates are only 12%-41%, even in patients who receive curative intent treatment[2].

Stage III NSCLC is the most clinically complex stage of lung cancer due to its heterogeneity. Although stage III disease is potentially curable, treatment outcomes depend on both treatment selection and timing (*i.e.* sequencing), which should be individualized according to the patient's unique clinical characteristics. Stage III NSCLC comprises a highly heterogeneous group of patients with varying degrees of local and nodal involvement. This heterogeneity is reflected in the 8th edition of the Tumor-Node-Metastasis lung cancer staging system, in which stage III disease is subdivided into three subgroups (stages IIIA, IIIB and IIIC)[2]. Thus, within stage III,

some patients could be eligible for upfront surgery (T3N1), while other patients could have potentially resectable disease after neoadjuvant therapy (up to stage T3 with non-bulky N2 involvement), and others might have unresectable tumors (most T4 tumors, bulky N2 disease, and N3).

For decades, the standard of care for patients with unresectable stage III NSCLC has consisted of a combination of platinum doublet-based chemotherapy and concurrent radiotherapy (RT), with a total dose of 66 Gy delivered in 33 daily fractions of 2 Gy/fraction (5 d/wk) for all histological variants, regardless of the molecular characteristics. Median progression-free survival (PFS) in these patients is 8-10 mo, and only 15% of patients survive for 5 or more years[3]. Several unsuccessful attempts have been made to improve long-term survival in these patients, including RT dose escalation and treatment intensification through intensity-modulated radiotherapy (IMRT)[4,5], chemotherapy (CT) consolidation strategies involving 2-3 additional cycles of CT after concomitant treatment[6], and targeted therapies such as cetuximab[7]. Given that the majority of recurrences after concomitant chemoradiotherapy (CRT) present with distant metastases, most research activity in recent years has centered on improving systemic control to improve cure rates[8].

Immune checkpoint inhibitors (ICIs) have proven effective in NSCLC. Initially, ICIs were positioned as second-line treatment and later as upfront palliative treatment for advanced disease. However, as the results of the PACIFIC trial show, ICIs also play an important role in locally advanced disease. Durvalumab is an anti-programmed cell death ligand 1 (PD-L1) human immunoglobulin (Ig) G1 monoclonal antibody that binds with high affinity and specificity to PD-L1 to block the interaction of PD-L1 with programmed cell death protein 1 (PD-1) and B7-1, avoiding inhibition of T-cell function, resulting in enhanced recognition and elimination of tumor cells by T cells. The PACIFIC trial is a double-blind, placebo-controlled, multicenter, phase III study conducted to evaluate the efficacy of durvalumab consolidation therapy after completion of concomitant CRT in patients with unresectable, stage III NSCLC without evidence of disease progression following concurrent CRT. This study demonstrated that 12 mo of treatment with consolidation durvalumab (10 mg/kg intravenous injection [iv] every 2 wk) after concomitant CRT in patients with unresectable stage III NSCLC significantly improved PFS and overall survival (OS), thus meeting an important unmet need after several years without any progress in treating this patient profile[9].

In this article, we describe the immunobiological basis for the benefits obtained by combining immunotherapy with RT in patients with NSCLC. We also review the updated data from the PACIFIC trial and related studies that have administered durvalumab in real-world clinical settings. This review takes a multidisciplinary approach to assessing the role of durvalumab, considering the perspectives of immunologists, pathologists, medical oncologists, and radiation oncologists to better understand the practical issues associated with the application of durvalumab in these patients. We also discuss how patient selection implies a critical need to modify work routines in order to successfully implement durvalumab therapy in routine clinical practice. Finally, we discuss the management of durvalumab-related toxicity and future challenges.

COMBINATION OF RADIATION THERAPY AND IMMUNOTHERAPY (ICI) IN STAGE III NSCLC: MECHANISMS OF ACTION

General immunotherapy concepts supporting the combined use of immunotherapy and RT

Anti-cancer immunotherapy, especially with ICIs, requires a different approach to conventional cancer treatment. While the main focus of surgery, CT, and RT is the direct elimination of tumor cells, the aim of immunotherapy is to help the body to restore an existing function to act against the tumor. Immunotherapy can help to "revive" the immune system's response capacity, which—theoretically—should have been sufficient to prevent tumor growth[10]. While several approaches to restoring immune function are available, ICIs are the most widely used.

Although immunotherapy alone has shown efficacy after failure of other treatments, the combination of agents that act on different pathways (*i.e.* on the tumor or the immune system) clearly increases the overall effectiveness of treatment[11]. Although resistance to treatments that directly target cancer cells (such as RT) should not, in theory, affect the impact of immunotherapy (due to differences in the mechanisms of

action), in reality the "collateral effects" of tumor-focused therapies on the immune system can alter the effects of immunotherapy, both positively and negatively.

Mechanism of ICI-based immunotherapy to consider when ICIs are combined with RT

Conceptually, although different ICIs (cytotoxic T-lymphocyte-associated protein 4 [CTLA4], PD-1, PD-L1, and in the future, T-cell immunoglobulin and mucin domain-3 [TIM-3] or V-domain Ig suppressor of T cell activation) will be prescribed depending on the specific target, all ICIs have the same goal: To restore the immune system's innate capacity to prevent tumor growth by blocking the function of inhibitory molecules[12]. In this regard, it is essential to avoid weakening the patient's existing immune function. Moreover, anything that strengthens the immune response (such as immunogenic cell death) is beneficial, as it helps to increase the anti-tumor effect of immunotherapy[13].

RT can influence the anti-tumor response in several ways, through three main mechanisms, which can produce either positive or negative effects. The first mechanism involves radiation-induced cell death, which can stimulate the release of tumor antigens or induce dysregulation in transcription, leading to increased expression of certain tumor neoantigens, which would not be expressed in unirradiated tumors; both of these effects strengthen the function of anti-tumor lymphocytes by eliminating tumor cells (immunogenic tumor death)[13]. In turn, these tumor antigens activate dendritic cells and cytokines, which recruit and generate an immune response that may have an anti-tumor effect. A second mechanism is the effect that RT has on suppressor cells (regulatory T cells, regulatory B cells, or myeloid-derived suppressor cells). In some cases, RT can eliminate these cells, although in most cases, these cells are highly resistant, and thus RT enhances local anti-tumor immunosuppression. The abscopal effect of RT confirms the distant effects of tumor irradiation (increased suppressor activity distant from the site of irradiation, which would not occur otherwise), and with it the increased immune response due to antigen release in the irradiated tissue, which could migrate—without "the brake"—to other non-irradiated tumors. A third effect of RT on immune response is to induce the production of cytokines and other membrane molecules, including checkpoint molecules such as PD-L1 or TIM-3. Considered together, these effects, which have been demonstrated experimentally[14], help to explain and support the growing interest in combining RT with ICIs to improve post-RT treatment response (Figure 1).

ICI-based immunotherapy combined with RT

At the physiopathogenic level, it is important to keep in mind that the mechanisms of tumor evasion go far beyond PD1-PD-L1/2 or CTLA4 pathways. The administration of a single ICI assumes that the targeted pathway plays a predominant role in tumor evasion, thus ignoring many other well-established mechanisms that also contribute to reducing the effectiveness of the immune system. If these mechanisms are not targeted therapeutically, they would reduce the effectiveness of immunotherapy. Fortunately, these different mechanisms can be targeted by administering various combinations of different ICIs (including molecules not clearly active in monotherapy such as TIM-3), an approach that is further supported by the optimal timing and form of RT, which also boosts immunotherapy[15,16].

The question of when and how to combine RT with ICIs needs to be defined to further consolidate this combination therapeutic strategy. However, if we take into account the mechanism of action, the optimal treatment sequence should be RT followed by administration of ICIs. In advanced cancers, it is important to avoid irradiating all tumor tissue in order to allow for a complete antigenic stimulation effect, which permits the lymphocytes to migrate to other tumor sites to seek out cancerous tissues, where they can act and amplify. In principle, it should take from 5 to 15 d for the lymphocyte response against the dendritic cells that have captured tumor antigens, causing the proliferation of anti-tumor T lymphocytes, which then locate the tumor while the ICI releases the natural blockade induced by the inhibitory molecules. Furthermore, RT also induces the release of free radicals and cortisol, which can have an immunosuppressive effect that typically reverts 7-15 d after RT (for this reason, the optimal approach—at least theoretically—is to postpone ICI administration for 1–2 wk)[16].

The clinical results of the PACIFIC trial further support the hypothesis regarding the synergistic effects of concomitant CRT and immunotherapy, which we describe in the following paragraphs.

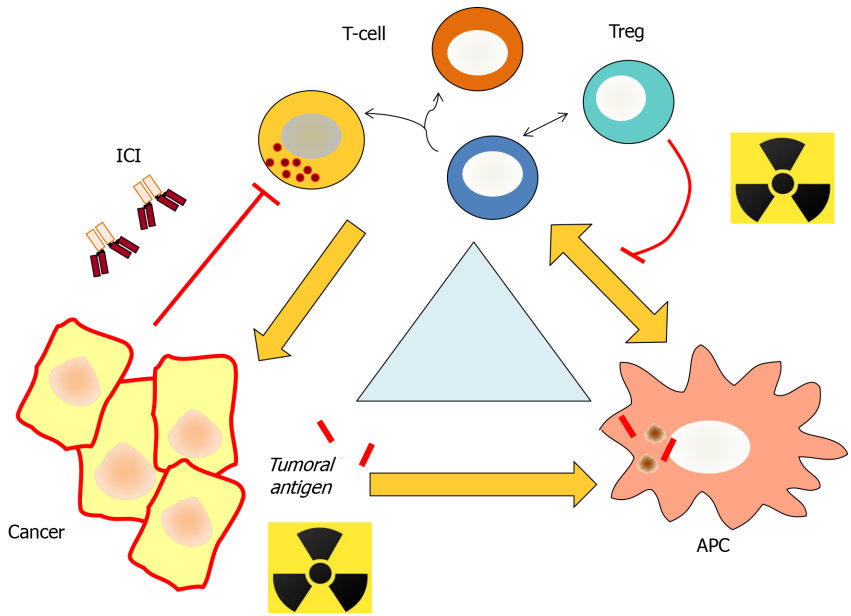

Figure 1 Radiotherapy and immune checkpoint inhibitors: Complementary mechanisms of action. The yellow arrows indicate anti-tumor processes and the red lines, mechanisms that block or suppress this anti-tumor immune response. APC: Adenomatous polyposis coli; ICI: Immune checkpoint inhibitors.

PACIFIC TRIAL AND IMMUNOTHERAPY-BASED CONSOLIDATION STRATEGY

The PACIFIC trial (NCT02125461) was a double-blind, placebo-controlled, multicenter, phase III clinical trial conducted in 253 centers in 26 countries on five continents. The trial was performed to evaluate the efficacy of 12 mo of durvalumab consolidation therapy administered intravenously at doses of 10 mg/kg every 14 d after completion of concomitant CRT in patients with unresectable, stage III NSCLC without evidence of disease progression following concurrent CRT. The primary endpoints were PFS (assessed by blinded independent central review) and OS in the intention-to-treat (ITT) population. The trial included 713 patients, who were randomized (2:1 ratio) to receive durvalumab or placebo starting 1 to 42 d after completing concomitant CRT. The patients were stratified according to age, sex, and smoking status. The initial analysis was performed in 2017 (median follow-up, 14.5 mo). Compared to placebo, durvalumab consolidation therapy significantly improved the following outcome measures: PFS (hazard ratio [HR] = 0.52, 95% confidence interval (CI), 0.42-0.65; $P < 0.0001$), response rate (28.4% *vs* 16%; $P < 0.001$), median duration of response (72.8% *vs* 46.8% at 18 mo), and median time to death or distant metastasis (23.2 mo *vs* 14.6 mo; $P < 0.001$)[17]. These findings were confirmed in an updated analysis in September 2018. At a median follow-up of 25.2 mo, durvalumab therapy significantly improved OS (HR: 0.68, 95%CI: 0.47-0.99; $P = 0.0025$), with a 24-mo survival rate of 66.3% in the durvalumab group (95%CI: 61.7-70.4) *vs* 55.6% in the placebo group (95%CI: 48.9-61.8). Median PFS in the active treatment group was 17.2 mo *vs* 5.6 mo in the placebo group (HR: 0.51, 95%CI: 0.41-0.63), and median survival until death or distant metastasis was 28.3 mo for durvalumab *vs* 16.2 mo for placebo (HR: 0.53, 95%CI: 0.41-0.68)[9].

Three-year survival outcomes were reported in February 2020 (median follow-up, 33.3 mo [range, 0.2-51.3]). At 3 years, the mortality rate was 44.1% in the durvalumab group *vs* 56.5% in the placebo group. OS rates for durvalumab *vs* placebo at 12, 24 and 36 mo were 83.1% *vs* 74.6%, 66.3% *vs* 55.3%, and 57.0% *vs* 43.5%, respectively. Treatment with durvalumab was associated with a 31% reduction in mortality risk (HR: 0.69 [95%CI: 0.55-0.86])[18]. These results strongly support durvalumab consolidation therapy as the standard of care in patients with unresectable, stage III NSCLC who do not progress after concomitant CRT. This indication has been approved by both the Food and Drug Administration (FDA) (February 2018) and the European Medicines Agency (EMA) (September 2018), although the latter limits its use to patients with PD-L1 expression in ≥ 1% of tumor cells, based on data from an unplanned post-hoc study. Scientific societies involved in the management of lung cancer recommend durvalumab consolidation treatment in these patients[19]. However, at present, the question of whether the benefits of durvalumab are limited to patients

with positive PD-L1 expression remains controversial, and it is not clear whether durvalumab treatment should be extended to patients with PD-L1-positive tumors < 1% and/or patients with unknown PD-L1 values.

Other clinical trials have been performed to assess the utility and safety of ICI-based consolidation immunotherapy after CRT in stage III NSCLC. In 2018, Durm *et al*[20] reported the results of a phase II study evaluating the use of pembrolizumab following CRT in 93 patients with unresectable stage III NSCLC. At a median follow-up of 18.6 mo, median time to metastatic disease or death (the primary endpoint) was 22.4 mo, with a median PFS of 17 and an estimated 2-year OS of 61.9%. In terms of adverse effects (AEs), 17.2% of patients developed grade ≥ 2 pneumonitis and 5.4% grade 3 or 4 pneumonitis. Another study, RTOG 3505 — a randomized phase III study of CRT followed by consolidation nivolumab (240 mg iv) or placebo every 2 wk for up to 1 year — was designed to assess survival efficacy and safety of nivolumab consolidation therapy after CRT with cisplatin and etoposide, but the trial was terminated early due to low enrollment (n = 20), due to publication of the PACIFIC findings[21]. Another multicenter, randomized, phase II study of consolidation immunotherapy with nivolumab and ipilimumab or nivolumab alone following CRT in this setting is currently enrolling patients (NCT 03285321). Yan *et al*[REF] reported safety results from the interim analysis of this study with first 20 patients accrued. Most toxicities were grade 1 or 2 and the most common grade 2 AEs included fatigue (25%), pneumonia (25%), and extremity pain (20%). The incidence of grade 3 or higher immune-related AEs was higher in the nivolumab/ipilimumab arm, but manageable according to the authors. No other studies have evaluated immunotherapy-based consolidation strategy after CRT; consequently, the PACIFIC trial is the first and only randomized phase III, placebo-controlled trial showing a benefit for both PFS and OS in this population to date (Table 1).

DETERMINATION OF PD-L1 PRACTICAL ISSUES

Requirements for optimal biological samples

Obtaining biological samples with optimal (or at least sufficient) quality to assess biomarkers should be a shared, multidisciplinary responsibility. For this reason, it is essential that all clinicians who treat these patients understand the advantages and disadvantages of each sample type. In addition, clear procedures should be established for ordering biomarker analyses and obtaining additional samples (if necessary). In this regard, it should be possible to automatically order new samples based on the findings of the pathologic report.

Accurate biomarker analysis requires a sufficiently large sample of tumor cells (≥ 50-100 viable cells for PD-L1)[22]. The sample must also meet the basic quality requirements for analysis[23], including guarantees to ensure that the time elapsed between extraction and fixation is as short as possible and that fixation is done in a 10% formalin-buffered solution for 6-12 h (small biopsies) or 24-48 h (surgical resections)[24]. In lung cancer, the most common samples used for diagnosis are small biopsies and/or cytological samples (cell blocks, smears, and liquid cytology). All of these are suitable for PD-L1 analysis, and the specific choice will depend on the experience and capacity of the individual laboratory[25]. The use of cytology samples to determine PD-L1 has not yet been validated, although a good correlation has been observed between cytology cell blocks and aspirate smears with biopsy[26].

PD-L1 as a predictive biomarker

PD-L1 is a type 1 transmembrane protein (B7-H1) belonging to the B7 family of ligands, which can be expressed in lymphocytes and tumor cells. In advanced NSCLC, PD-L1 overexpression is a predictor of clinical benefit with PD-1/PD-L1 inhibitors[27]. The EMA limits the use of consolidation durvalumab to patients with positive PD-L1 expression (≥ 1% of tumor cells) based on the results of the PACIFIC trial[9].

Various immunohistochemical methods involving antibody clones against PD-L1 are available, the most common being 22C3 and 28-8 (Agilent/Dako), which share the Autostainer Link48 platform, and SP263 (MedImmune/Ventana) and SP142 (Spring/Bioscience/Ventana), which share the Ventana BenchMark platform. Using these four clones, PD-L1 is evaluated as the percentage expression in tumor cells (partial or complete membrane expression) at any intensity; SP142 assess the proportion of the stromal area occupied by immune cells with PD-L1 expression. Three methods—22C3, 28-8, and SP263—have all demonstrated technical equivalency for the determination of PD-L1 expression in tumor cells in patients with lung

Table 1 Overview of the results of overall survival and progression-free survival efficacy of immunotherapy based-consolidation studies (PACIFIC, LUN 14-179) and historic attempts to improve overall survival in stage III non-small cell lung cancer (PROCLAIM and RTOG 0617)

Endpoint	Study and treatment					
	PACIFIC durvalumab	PACIFIC placebo	LUN 14-179 pembrolizumab	PROCLAIM pemetrexed	RTOG 0617 (60 Gy RT)	RTOG 0617 cetuximab
Median follow-up	33.3 mo	33.3 mo	18.6 mo	22.2 mo	22.9 mo	21.3 mo
OS						
Median	NR	29.1 mo	22.4 mo	26.8 mo	28.7 mo	25 mo
12-mo	83.1%	74.6%	74.7%	76%	80%	76.2%
24-mo	66.3%	55.3%		52%	57.6%	52.3%
36-mo	57%	43.5%		40%		
PFS						
Median	17.2 mo	5.6 mo	17 mo	11.4 mo	11.8 mo	10.8 mo
12-mo	55.9%	35.3%	60.2%		49.2%	44.3%
24-mo			44.6%		29.1%	24.2%

OS: Overall survival; PFS: Progression-free survival; RT: Radiotherapy.

cancer[27].

Several studies have examined differences in PD-L1 expression between the primary tumor and metastatic lesions[27]. The general consensus is that PD-L1 expression is heterogeneous in 25% of cases, both within the tumor itself and in the different tumor sites, including regional lymph nodes. Studies have shown that treatment with CT or RT increases PD-L1 expression[28]. In this context, large, representative biological samples of the tumor are needed to accurately perform the analysis, and the general recommendation is to analyze pre-treatment samples from the primary tumor and to ensure that additional samples are available as an alternative if this proves inadequate.

Quality assurance in PD-L1 determination

When determining the presence of any biomarker (including PD-L1), it is essential to ensure the quality of the results by strictly following laboratory policies. In Europe, the testing laboratory should have ISO 9001 certification and all tests should be accredited according to the UNE-EN ISO15189 standard. This quality policy assumes that trained personnel (technicians, biologists, pathologists) are available and that they adhere to standardized work procedures, using well-maintained instrumentation with CE certification, as well as validated reagents[29]. The use of internal controls for each assay is important, and the laboratory itself should also undergo external quality controls (*e.g.*, SEAP, EMQN, UK-NEQAS). It is also advisable to check the results to verify that the percentage of positive findings is consistent with published reports.

Laboratory results report

The laboratory report should also meet certain quality parameters, including the following: (1) Recommended response time: 7-10 business days; (2) Compliance with the quality policies described above; and (3) Inclusion of the following data points: Identification of the patient and the person requesting the test; pathological diagnosis; sample type; sample collection and processing date; anatomic origin of sample; date of request, reception, and issuance of the result; type of assay used and description of the limitations; for commercial assays, the following information must be included: Commercial name; batch number; confirmation that the assay is approved for *in vitro* diagnosis; description of the sample quality; adequacy of the sample; test result given as the percentage of tumor cells with PD-L1 expression; identification of the professional(s) responsible for carrying out the test and (optionally) the name of the laboratory supervisor; any additional information or comment of interest; and, accreditation, certification, or participation in quality assurance programs.

REAL-WORLD DATA FOR DURVALUMAB CONSOLIDATION THERAPY

To date, only two studies (both retrospective) have been carried out to evaluate consolidation durvalumab in a real-world clinical setting. Preliminary data from these studies were reported at the 2019 World Conference on Lung Cancer. Grivet *et al*[30] reported results from a retrospective cohort of 83 patients in Brazil with locally advanced NSCLC who were treated with durvalumab. The cohort was comprised mainly of females (55%) and most patients were smokers (59%), with a median age of 73.5 years. The most common histological finding was adenocarcinoma (67%). The most common chemotherapy regimen was carboplatin-paclitaxel, and the main RT scheme was 60 Gy in 30 fractions (39%). After a median of 1.2 years on durvalumab, only 8 patients had died (9.6%), and none of these deaths was considered treatment-related. The main toxicities were mucositis (63%), cough (52%), and cutaneous rash (48.8%). No cases of grade 5 toxicity were reported. At the same congress, Barbaro *et al*[31] reported results from a retrospective cohort of 146 patients who had completed concomitant CRT for locally-advanced NSCLC at the Montefiore Medical Center from 2007-2018. Of these patients, 27% did not meet PACIFIC eligibility criteria for durvalumab therapy, mainly due to the presence of concurrent diseases (32%) or additional malignancies (15%); other reasons were disease progression (7%) and pneumonitis (7%). Thus, in the full cohort, only 17 patients received durvalumab, including four who did not meet criteria for durvalumab (due to co-morbidities and/or additional malignancy). The median time to durvalumab initiation following CRT was highly variable, ranging from 56 d prior to durvalumab approval in July 2018 to 30 d afterwards. Although the follow-up was short, there was a trend toward improved OS at 15-mo in patients who received durvalumab *vs* those who did not (100% *vs* 87.5%).

The PACIFIC-Real World (PACIFIC-R) trial is currently in progress. The aim of this study is to assess the efficacy and safety of durvalumab treatment after concurrent CRT in a large, real-world population. The study plans to enroll approximately 1200 NSCLC patients through early access programs between 2017 and 2018, with a 5-year follow-up. Interestingly, in this study patients will be enrolled regardless of tumor PD-L1 expression level. Primary endpoints are PFS (investigator-assessed) and OS.

WHICH PATIENTS SHOULD BE INCLUDED OR EXCLUDED FROM THE PACIFIC REGIMEN?

In the PACIFIC trial, the main eligibility criteria were locally advanced, stage III unresectable NSCLC patients treated with definitive concurrent CRT (54 to 66 Gy; V20 < 35%). Additional inclusion criteria were: (1) No progression after CRT; (2) Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 1; and (3) Last radiation dose administered from 1 to 14 d before randomization (after a protocol amendment, this criterion was modified to 1 to 42 d before randomization). Unresolved toxicity ≥ grade 2 and pneumonitis ≥ grade 2 from previous treatment with CRT were the main exclusion criteria. These criteria suggest that some patients will not be eligible for durvalumab therapy after CRT. Currently, published data regarding eligibility criteria in real-life clinical practice area limited; however, a few retrospective, single-center studies have specifically assessed these criteria. Sakaguchi *et al*[32] evaluated the clinical criteria for durvalumab after CRT in an historical cohort of 73 patients with unresectable stage III NSCLC based on the eligibility criteria established in the PACIFIC trial. This patient cohort was treated between 2011 and 2018 and approximately 30% did not meet eligibility criteria for durvalumab therapy. The most common reasons for ineligibility were radiation pneumonitis (16% of cases), poor PS (10%), and disease progression after CRT (4%). Unfortunately, no data on the V20 or other factors related to radiation quality and risk of pneumonitis were reported.

In another cohort involving 97 patients with stage III unresectable NSCLC patients treated between 2017 and 2019, Shaverdian *et al*[33]explored the clinical limitations for durvalumab therapy. Durvalumab was not administered in approximately 27% of patients, similar to the results described by Sakaguchi and colleagues. The main exclusion criteria were disease progression and/or CRT-related toxicity. All patients underwent pre-treatment staging with brain magnetic resonance imaging (MRI) and positron emission tomography-computed tomography (PET-CT) to ensure that only stage III patients were included in the study. In contrast to the study by Sakaguchi *et al*[32], Shaverdian and colleagues reported PTV values and cardiac doses. Consistent with these two retrospective studies, Barbaro *et al*[31] (see section 4) found that around

27% of patients were not eligible for durvalumab according to PACIFIC criteria. Although these data were obtained in retrospective studies and small cohorts, they consistently show that approximately one out of every 3 or 4 patients (25%-33%) are not eligible for durvalumab therapy after CRT.

Clinical considerations

Lung cancer is most commonly diagnosed in older people, which may be a limiting factor for the prescription of durvalumab, as some published reports suggest that durvalumab may be more toxic in older patients[34]. In this regard, Socinski *et al*[35] analyzed data from patients ≥ age 70 included in the PACIFIC trial, who accounted for 22% of the full cohort. Most of these older patients were males with ECOG PS 1 treated with carboplatin-based CT. However, this subgroup analysis did not find any age-related difference in median survival outcomes. In the < 70 years subgroup, median survival in the treatment arm was 16.9 mo *vs* 5.6 in the placebo arm (HR: 0.53, 95%CI: 0.42-0.67); similarly, in patients ≥ age 70, survival in the treatment arm was 12.3 *vs* 6.1 mo (HR: 0.62, 95%CI: 0.41-0.95). However, in the patients who received durvalumab, older patients had higher rate of serious AEs (42.6% *vs* 24.9%) and grade 3/4 AEs (41.6% *vs* 29.4%), but fewer adverse events of special interest (56.4% *vs* 67.9%) than younger patients.

In terms of OS, subgroup analyses in the PACIFIC trial showed a greater benefit for durvalumab in certain groups: Females benefitted more (HR: 0.46; 95%CI: 0.3-0.73) than males (HR: 0.78; 95%CI: 0.59-1.03), as did Caucasians (HR: 0.71; 95%CI: 0.54-0.92) *vs* Asians (HR: 0.62; 95%CI: 0.38-1.01), and American patients (HR: 0.46; 95%CI: 0.3-0.69) *vs* Asians (HR: 0.67; 95%CI: 0.41-1.11) and Europeans (HR: 0.66; 95%CI: 0.61-1.21)[9].

Biomarkers: PD-L1 expression

The PACIFIC trial enrolled patients regardless of tumor PD-L1 expression level. However, a *post hoc* exploratory analysis performed according to PD-L1 expression (available in 63% of the overall cohort; n = 451)[36] found that 35% had ≥ 25% PD-L1 expression, 67% had ≥1% PD-L1, and 33% were negative (< 1%). Median PFS was higher in the durvalumab arm *vs* placebo across all these subgroups, as follows: In the subgroup with ≥ 25% PD-L1 expression: 17.8 *vs* 3.7 mo (HR: 41, 95%CI: 0.26-0.65); in the ≥ 1% PD-L1 group: 17.8 *vs* 5.6 mo (HR: 0.46, 95%CI: 0.33-0.64); in the negative (< 1% PD-L1 expression) subgroup: 10.7 *vs* 5.6 mo (HR: 0.73, 95%CI: 0.48-1.11). Finally, in patients with unknown PD-L1 expression, median PFS was 14 mo *vs* 6.4 mo (HR= 0.59, 95%CI: 0.42-0.83). Based on this subanalysis, in July 2018 the EMA approved durvalumab for patients diagnosed with locally-advanced, unresectable stage III NSCLC who have not progressed following CRT, only for patients whose tumor PD-L1 expression is ≥ 1%. This restricted approval, which differed from the FDA approval (which did not require any minimum PD-L1 expression level) remains controversial in the European oncology community because it means that patients with low and unknown PD-L1 expression are unable to benefit from the improved survival outcomes that consolidation durvalumab provides. In this regard, it is worth noting that the PACIFIC trial found a survival benefit in patients with unknown PD-L1, who comprised 36.7% of the study population[9]. Nevertheless, several ongoing clinical trials will establish the true value of PD-L1 status in these patients[37].

Treatment considerations

Durvalumab consolidation therapy improved both PFS and OS, irrespective of the type of chemotherapy prescribed, the radiation dose, or time from RT to durvalumab initiation. However, to our knowledge, no large, clinical studies have yet examined all of these variables in a real-world setting.

Chemotherapy-based considerations: The PACIFIC trial showed that several different chemotherapy strategies can be concurrently administered with RT. In that trial, patients received ≥ 2 cycles of platinum-based chemotherapy (etoposide, vinblastine, vinorelbine, a taxane, or pemetrexed) concurrently with definitive RT. Induction chemotherapy (ICT) followed by concurrent CRT was compared to two cycles of chemotherapy delivered concurrently with RT[17]. Spigel *et al*[38] evaluated the impact of ICT in the PACIFIC trial. Overall, 26% and 29% of patients in the durvalumab and placebo groups, respectively, received ICT. Durvalumab demonstrated a clinical benefit in both groups (with and without ICT) and this was not influenced by the specific ICT regimen. Although safety outcomes were similar (with only minimal differences) across subgroups, the toxicity rate (including pneumonitis) was lower in patients who received ICT, regardless of the treatment arm.

Another important factor to consider is the CRT scheme. Even though concomitant CRT improved outcomes when compared to sequential CRT[39], some patients receive sequential CRT, generally due to the presence of frailty, certain comorbidities, or poor performance status. This subset of patients was not enrolled in the PACIFIC trial, so there is still a lack of evidence for the benefit of durvalumab in this setting. Interestingly, the currently ongoing phase 2 PACIFIC-6 trial (NCT03693300) was designed to assess safety, efficacy, and quality of life (QoL) in 150 patients treated with sequential CRT followed by durvalumab. The primary objective of that study is safety, with secondary endpoints that include PFS, OS, duration of response, response rate, among others. Interestingly, patients with ECOG PS 2 are eligible and there are no biomarker-based enrolment limitations (*e.g.*, PD-L1 expression).

Radiation therapy-based considerations: In the last few years, technological and methodological innovations have improved the accuracy and efficacy of RT. However, RT delivery requires an experienced team and high-quality control measures to ensure that patients receive the most accurate and effective treatment possible to improve clinical outcomes[40].

PET-CT staging is recommended for patients with NSCLC considered candidates for radical RT, as this imaging modality permits accurate staging of stage III NSCLC to better define intrathoracic nodal disease for RT planning, especially compared to conventional staging techniques[41]. Moreover, PET-CT can also detect occult metastatic disease in up to 24% of cases[42]. However, in the PACIFIC trial, PET-CT staging was not mandatory, so some patients with stage IV disease could have been enrolled inadvertently.

Radiation therapy quality assurance (RTQA) is also important, as this ensures the use of high-quality methodologies and procedures to efficiently achieve quality RT. The absence of RTQA has been associated with major violations in clinical trials, with a negative impact on patient outcomes, as reported in the PROCLAIM study[43]. Unfortunately, in the PACIFIC trial, no real-time RTQA quality control was centrally performed, and not all relevant data could be collected after the trial ended.

At present, there are only limited data on durvalumab consolidation in real-world settings, and there are important questions regarding the benefit of the PACIFIC strategy in routine clinical practice. We are certain that large, real-world studies will provide more information about the efficacy of consolidation durvalumab in different subgroups to better determine the patient population most likely to benefit from this therapy. Additionally, such studies could provide an extraordinary source of data to delve deeply into the methodological limitations and quality control issues related to RT that we have emphasized in this review, to fully elucidate the real magnitude of the benefits and risks (*e.g.*, pneumonitis) of durvalumab. In this regard, several ongoing phase 2 and 3 clinical trials (Pacific-R, PACIFIC -2, Pacific-5, Pacific-6) are expected to resolve these open questions.

DESCRIPTION AND MANAGEMENT OF TOXICITY ASSOCIATED WITH THE PACIFIC REGIMEN

The toxicity profile was a secondary endpoint in the PACIFIC trial. In general, tolerance to consolidation durvalumab after concomitant CRT was good, with no increase in general toxicity in the active treatment group *vs* placebo. Adverse event rates of any grade were 96.8% and 94.9% in the durvalumab and placebo groups, respectively; the corresponding rates of grade 3/4 toxicity were 30.5% and 26.1%. However, a higher proportion of patients in the durvalumab group had to interrupt treatment due to toxicity (15.4% *vs* 9.8%). The most common treatment related toxicities are listed in Table 2. The most frequent AEs leading to treatment interruption were pneumonitis, radiation pneumonitis, and pneumonia. The incidence of radiation pneumonitis or pneumonitis was significantly higher in the durvalumab group (33.9% *vs* 24.8%), but no significant between-group differences were observed in terms of grade 3/4 pneumonitis (3.4% *vs* 2.6%). Serious AEs were more common in the durvalumab group (29.1% *vs* 23.1%), but the mortality rate was significant higher in the placebo group (6.4% *vs* 4.4%)[44].

One of the main concerns of administering ICIs in patients treated with radical thoracic RT is the potential for an increased risk of pneumonitis, as studies show that a high percentage of patients receiving radical intent, concomitant thoracic RT develop some degree of pneumonitis[32]. In the phase I KEYNOTE-001 trial carried out to evaluate pembrolizumab in patients with advanced NSCLC, a higher rate of ICI-

Table 2 Treatment-related events reported in ≥ 11% of patients in PACIFIC study arms and their rates in LUN 14-179 study[1]

Event	Treatment arm and study					
	Durvalumab in PACIFIC, *n* = 475		Placebo in PACIFIC, *n* = 234		Pembrolizumab in LUN 14-179	
	Any grade	Grade 3/4	Any grade	Grade 3/4	Any grade	Grade 3/4
Any event	96.8%	30.5%	94.9%	26.1%		
Cough	35.2%	0.4%	25.2%	0.4%	25.8%	1.1%
Fatigue	24%	0.2%	20.5%	1.3%	46.2%	4.3%
Dyspnea	22.3%	1.5%	23.9%	2.6%	21.5%	5.4%
Radiation pneumonitis	20.2%	1.5%	15.8%	0.4%		
Diarrhea	18.5%	0.6%	19.7%	1.3%	15.1%	4.3%
Pyrexia	15.2%	0.2%	9.4%	0%		
Nausea	14.3%	0%	13.2%	0%	15.1%	1.1%
Anorexia	14.3%	0.2%	12.8%	0.9%	17.2%	1.1%
Pneumonia	13.3%	4.4%	7.7%	3.8%		
Pneumonitis	12.6%	1.9%	7.7%	1.7%	17.2%[1]	5.4%
Arthralgia	12.4%	0%	5.1%	0%	15.1%	1.1%
Pruritus	12.4%	0%	5.1%	0%	10.8%	0%
Rash	12.2%	0.2%	7.7%	0%	12.9%	1.1%
Constipation	11.8%	0.2%	8.5%	0%		
Hypothyroidism	11.6%	0.2%	1.7%	0%	7.5%	0%

[1]Grade ≥ 2.

related thoracic toxicity was observed in patients previously treated with thoracic RT *vs* those who had not received prior RT (13% *vs* 1%, *P* = 0.046)[45]; however, some data suggest that anti-PD-L1 treatments (such as durvalumab) may have a lower risk (compared to anti-PD-1 treatments) of lung toxicity[46]. In general, the addition of consolidation durvalumab to concomitant CRT was accompanied by low rates of severe lung toxicity, a finding that suggests the benefit of durvalumab outweigh the risks. Moreover, this implies that there is no need to restrict the use of durvalumab due to pulmonary toxicity, although it should be noted that the thoracic RT doses in the PACIFIC trial were lower than usual (up to 54 Gy)[47].

In a subgroup analysis performed to identify predisposing factors for pneumonitis in the PACIFIC trial, the pneumonitis rate was higher in Asians (47.9% *vs* 17.6%) and in patients with EGFR mutations (11% *vs* 3.8%). No significant associations were found between pneumonitis and other respiratory disorders, the previous RT dose, or the type of CT received (cisplatin *vs* carboplatin)[48]. However, we lack data from large studies to confirm the absence of predisposing factors for pneumonitis, which could potentially limit the use of consolidation durvalumab therapy. In the PD-L1 subgroup analysis in the PACIFIC trial, any grade pneumonitis was more common in patients treated with durvalumab in all subgroups (ranging from 30.8% to 35.7%) *vs* placebo (17.4% to 29.9%). However, there were no significant differences in the incidence of grade 3 or 5 pneumonitis (no grade 4 events were reported)[36].

As mentioned above, very few real-world studies of durvalumab have been conducted. However, one multicenter study (*n* = 36) found that 29% of patients developed symptomatic pneumonitis (grade ≥ 2), with only two cases (5.6%) of grade 3 pneumonitis. In that study, the median time to pneumonitis was 71 d (range, 29-270). The mean duration of pneumonitis was 6 wk (range, 2-19), and the median time to durvalumab interruption was 4.5 wk (range, 2-8). However, 70% of these patients restarted durvalumab after the pneumonitis resolved, with a recurrence rate of 14%. The presence of pneumonitis did not affect survival outcomes[49].

Based on the consensus guidelines and recommendations of the European Respiratory Society, Society for Immunotherapy of Cancer, American Society of Clinical Oncology, and the European Society of Medical Oncology, immune-mediated

pneumonitis derived from durvalumab should be graded based on CTCAE Version 5.0 criteria[50]. We recommend the following management approach: Grade 1: Continue treatment with durvalumab but with close follow-up; Grade 2: Interrupt durvalumab therapy and maintain close follow-up. Initiate corticosteroid therapy (oral methylprednisolone 1mg/kg per day or equivalent). Consider restarting durvalumab after resolution or downgrade to grade 1; Grade ≥ 3: Discontinue durvalumab permanently and initiate very high dose corticosteroid therapy (methylprednisolone 2-4 mg/kg per day IV or equivalent) +/- bolus intravenous steroid. If symptoms do not improve within 48 h or clinical worsening is observed, consider infliximab 5 mg/kg, cyclophosphamide 600 mg/m^2, or mycophenolate mofetil 1000 mg/12 h.

In cases with symptomatic pneumonitis, steroid treatment, once started, should be maintained for ≥ 6 wk to avoid recurrences. Steroid treatment should be gradually tapered over time[51,52].

QUALITY OF LIFE IN THE PACIFIC TRIAL: PATIENT-REPORTED OUTCOMES

In the PACIFIC trial, patient-reported outcomes (PROs) were pre-specified secondary outcomes[14]. In the ITT population, the investigators evaluated PRO symptoms, functioning, global health status, and QoL using the scales most commonly administered in cancer patients (EORTC Quality of Life Questionnaire-Core 30 [QLQ-C30] and the lung cancer version, the QoL-Lung Cancer 13 [QLQ-LC13]). These scales were administered at various time points, including at baseline (randomization), every 8 wk until week 48, and then every 12 wk until progression. In addition, the investigators also assessed changes in key symptoms from baseline to 12 mo (changes ≥ 10 points were defined as clinically relevant), with mixed model for repeated measures (MMRM) and time-to-event analyses.

Hui *et al*[53] recently reported the results of the PROs analysis. No significant changes were observed in the two treatment arms in the pre-specified longitudinal PROs of interest: Cough (MMRM-adjusted mean of 1.8 with durvalumab *vs* 0.7 with placebo), dyspnea (3.1 *vs* 1.4), chest pain (-3.1 *vs* -3.5), fatigue (-3.0 *vs* -5.2), appetite loss (-5.8 *vs* -7.0), and physical functioning (0.1 *vs* 2.0). In both treatment arms, the global health status/QoL (2.6 with durvalumab *vs* 1.8 with placebo) remained stable, with no clinically significant variations from baseline. Additionally, no clinically relevant between-group differences were observed in changes from baseline to 12 mo and in time to deterioration in these key PROs. Thus, the durvalumab benefit did not affect overall health and QoL, with no detrimental impact observed in the main indicators of PROs.

MANAGEMENT OF STAGE IIIA PATIENTS AFTER PUBLICATION OF THE PACIFIC RESULTS

Patients with NSCLC classified as stage IIIA due to the presence of mediastinal lymph node involvement (N2) may present differences in the number of nodal stations involved, their size, and/or the existence of capsular rupture. These factors can all influence the prognosis and thus the treatment strategy[54]. Therefore, it is evident that this group of patients is comprised of different subgroups with important differences that can require different treatment strategies. The specific treatment approach should ideally be based on a consensus agreement reached by a multidisciplinary tumor board.

Based on the findings of the PACIFIC trial, 12 mo of durvalumab consolidation therapy is now considered the standard treatment for patients with unresectable stage IIIA NSCLC, provided that no contraindications for immunotherapy are present. For patients with resectable disease, the optimal treatment strategy remains controversial. Upfront surgical resection is indicated in patients without mediastinal lymph node involvement (N0-N1) in whom complete resection is technically feasible (provided the patient's general condition and lung function allow it). In these patients, the treatment indication is adjuvant platinum doublet chemotherapy, as this has been shown to significantly prolong OS[55]. Adjuvant RT should be reserved for patients with positive surgical margins and postoperative mediastinal node involvement[56]. RT should, preferably, be administered sequentially after CT, except in cases with macroscopic residual disease[39,57]. The role of surgery in patients with stage IIIA disease (nodal

involvement) is more controversial; in most cases, concomitant CRT is considered the standard option. However, in patients with a single involved mediastinal node < 3 cm with evidence of radiological response after CT or induction RT, surgical resection would be a valid option, although prospective randomized trials have yet to find any difference between these two treatment strategies[39,58]. The data from the PACIFIC trial add to this controversy: Since durvalumab consolidation therapy has been shown to significantly improve OS *vs* concomitant CRT alone, durvalumab should also be considered the standard of care in stage IIIA disease. That said, this approach could change in coming years based on the highly promising initial data from clinical trials evaluating the role of neoadjuvant immunotherapy combined with CT in patients with resectable (or potentially-resectable) stage III NSCLC. Forde *et al*[59], in a study involving 23 patients with stage I-IIIA disease who received nivolumab monotherapy, reported a high pathological response rate (45%) with no correlation between PD-L1 expression and efficacy. More recently, Provencio *et al*[60] reported the results of the NADIM trial, a multicenter phase II clinical trial evaluating nivolumab combined with carboplatin-based CT and neoadjuvant paclitaxel, reporting an even higher pathological response rate (84.6%). If these data are confirmed in large prospective studies, we will need to reopen the debate regarding induction therapy followed by surgery *vs* concomitant CRT followed by durvalumab consolidation therapy. In any case, it is evident that immunotherapy with PD-1/PD-L1 inhibitors will, one way or another, be incorporated into the treatment regimen for patients with stage IIIA NSCLC.

MODIFICATION TO CLINICAL ROUTINES: IMPLEMENTING THE PACIFIC REGIMEN IN REAL LIFE

The emergence of durvalumab consolidation therapy in locally advanced disease represents a new challenge for routine clinical practice, requiring clinicians to implement a series of changes in the diagnostic, treatment, and follow-up processes of these patients (Figure 2).

Changes in the diagnostic process

The radiological protocols to rule out distant metastasis remain unchanged, and include PET-CT, brain MRI/CT, and mediastinal staging (endobronchial ultrasonography (EBUS)/esophageal ultrasonography (EUS) or the more invasive technique, mediastinoscopy). By contrast, pathological evaluation has become more important. In the histological analysis, a diagnosis of NSCLC would be sufficient, regardless of the tumor histology (squamous or non-squamous), as the PACIFIC trial demonstrated the efficacy of durvalumab in both histological groups[14].

The EMA authorization limited consolidation durvalumab therapy to patients with PD-L1 expression ≥ 1%, obliging the European cancer care community to intensify efforts to evaluate the role of this biomarker in all patients with unresectable stage III NSCLC. This limitation also increases the need to ensure that adequate tumor sample are obtained and to make use biopsy rather than cytology for the diagnosis, particularly in centers that do not have the capability for PD-L1 determination in cytological samples. Consequently, in some cases the tests (bronchoscopy, EBUS, CT-guided biopsy) will need to be repeated or, alternatively, surgical procedures (the least invasive methods available) will be necessary for the diagnosis.

One near-term objective is to validate cytological samples (*e.g.*, cell block) for the analysis of PD-L1. This would increase the proportion of patients with PD-L1 determination and, in turn, limit the number of diagnostic tests, thus reducing costs, saving time, and also decreasing the risks that these procedures pose for the patient.

Although the PACIFIC trial included patients with and without molecular alterations in EGFR or ALK, immunotherapy is of limited benefit in these patients[40]. However, if the studies currently underway in this population (LAURA, NCT NCT03521154) show a benefit for the use of targeted therapies, we will need to determine the patient's mutational status to decide whether or not the patient is a candidate for durvalumab therapy.

Changes in patient management and follow-up

Goal—patient safety: Pneumonitis is one of the most limiting and well-known toxicities of concomitant CRT. The reported incidence of pneumonitis in the PACIFIC trial was approximately 15% (severe pneumonitis: < 5%); however, this could be

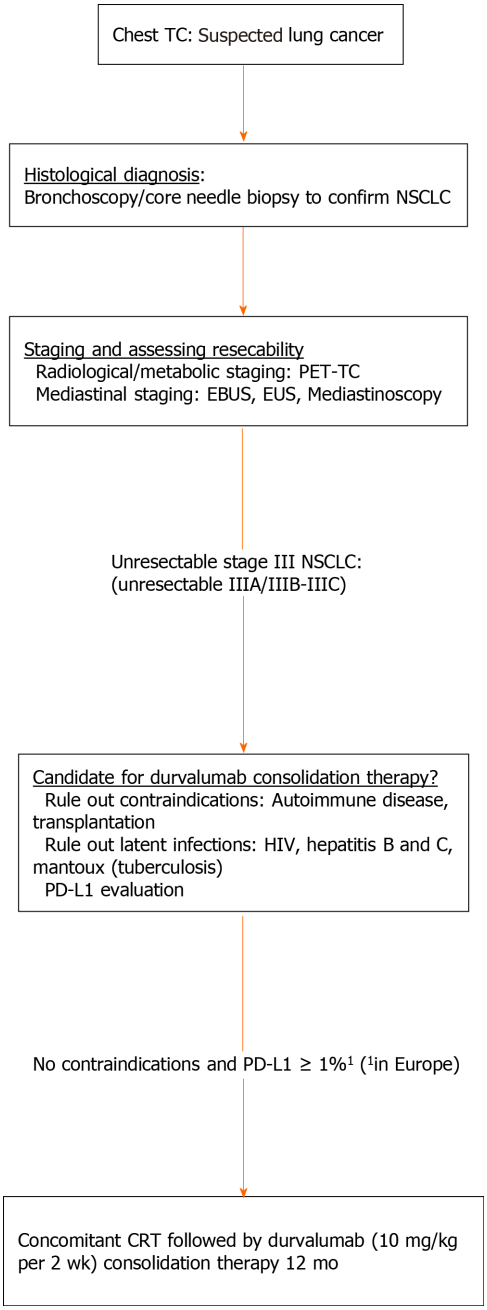

Figure 2 Decision making process diagram to evaluate candidate patients for durvalumab consolidation according to PACIFIC trial strategy in clinical practice. [1]In Europe. CRT: Chemoradiotherapy; EBUS: Endobronchial ultrasonography; EUS: Esophageal ultrasonography; HIV: Human immunodeficiency virus; NSCLC: Non-small cell lung cancer; PET-CT: Positron emission tomography-computed tomography; PD-L1: Programmed cell death ligand 1.

higher in real-world clinical settings due to the presence of respiratory comorbidities[43,44]. Consequently, baseline respiratory function should be assessed at least once between completion of RT and initiation of durvalumab therapy. In addition, respiratory function should be periodically monitored by a pulmonologist during treatment and follow-up.

Immunotherapy trials conducted to date have systematically excluded patients with chronic infections (hepatitis B and C, human immunodeficiency virus [HIV], and tuberculosis [TB]) and risk of reactivation. The risk *vs* benefits of durvalumab in patients with localized disease is unknown, so these infections should be ruled out before initiating treatment. The most common and widely available techniques for this purpose are viral serology (hepatitis and HIV) and the Mantoux test for TB.

Thyroid alterations (thyroid stimulating hormone [TSH], T3, and T4) are common in patients treated with ICIs. For this reason, a baseline determination of these hormones should be performed and their levels measured every 4-6 wk during treat-

ment. Autoimmune diseases should be ruled out to ensure patient safety.

Objective—radiological evaluation: In the PACIFIC trial, early initiation of durvalumab after concomitant CRT (≤ 14 d) was associated with a trend towards higher OS. Chest CT should be performed as soon as possible after completion of RT (preferably < 4 wk): Importantly, the role of chest CT is not to assess response, but rather to rule out local progression and/or signs of severe pneumonitis, which would contraindicate consolidation durvalumab therapy.

The emergence of nodal involvement or enlarged nodes during follow-up without other signs of progression should be confirmed histologically. Granulomatous/sarcoid-like lesions in the lymph nodes have been described in patients treated with immunotherapy[45], potentially requiring specific management or treatment.

FUTURE DIRECTIONS

After publication of the PACIFIC trial results, numerous questions have been raised regarding how to optimize immunotherapy in stage III NSCLC in terms of treatment duration, combinations, and RT schemes. This has even prompted a search for new biomarkers or additional treatments.

Treatment duration

Based on the data from the PACIFIC trial, the recommended duration of durvalumab therapy is 1 year. There may be a subset of patients (*e.g.*, with low tumor burden, better radiological or metabolic response) who could obtain a similar benefit with a shorter duration of treatment, although data to support this approach are lacking.

Optimal timing and immunotherapy treatment scheme

RT and PD-L1 inhibitors have a synergistic effect that creates a more immunogenic tumor microenvironment by promoting CD8 T lymphocyte infiltration and suppression of suppressive myeloid cells[61]. Two prospective studies are currently evaluating the combined administration of anti-PD-1/PD-L1 agents during concomitant CRT: PACIFIC-2 (NCT03519971) with durvalumab and KEYNOTE-799 (NCT03631784) with pembrolizumab. However, the potential toxicity of these combinations will need to be observed carefully given findings from previous studies with nivolumab[62] and pembrolizumab[63], both of which showed highly promising results but with an increased risk of pneumonitis. Atezolizumab combined with concomitant CRT is being evaluated in the DETERRED study[64], which will also determine the efficacy and safety of this anti-PD-L1 agent combined with chemotherapy as a consolidation strategy after concomitant CRT.

Although concomitant CRT is the standard treatment in stage III NSCLC, there are some cases—due to the presence of comorbidities, the patient's functional situation (poor PS and high risk of toxicity)—in which sequential treatment (CT followed by RT) is preferred. As consolidation durvalumab therapy becomes more widely used, the evidence base for sequential treatment in these patients has increased, but prospective data remain unavailable. The prospective, randomized PACIFIC-5 trial (NCT03706690) is currently underway to compare concomitant to sequential therapy. Moreover, that study includes monthly administration of durvalumab (1500 mg iv), which will provide novel efficacy and safety data for that dose level.

Other studies are investigating combined treatment with RT and immunotherapy in which chemotherapy is excluded. The SPRINT study is currently evaluating the efficacy of induction pembrolizumab monotherapy and maintenance RT in patients with PD-L1 > 50%[65]. The DUART study will evaluate the efficacy and safety of durvalumab after RT in patients not eligible for chemotherapy (NCT04249362). Other alternative strategies include combinations with drugs with different mechanisms of action (*e.g.*, anti-CTLA-4) (NCT03663166).

Alternative RT schemes

At present, due to its proven efficacy and safety, the standard RT scheme is a total dose of 60 Gy at 2 Gy/d[7].Alternative approaches such as hypofractionated RT combined with immunotherapy are being evaluated in the NRG LUN 004 trial (NCT03801902). The combination of immunotherapy with proton therapy could reduce the cardiopulmonary toxicity associated with conventional RT, thus increasing the safety of hypofractionated schemes. This approach is being prospectively evaluated in another trial (NCT03818776).

Predictive biomarkers and PD-L1

At present, access to durvalumab consolidation in Europe is limited to patients with positive PD-L1 expression (> 1%). In addition to the well-known limitations of PD-L1 as a biomarker (interobserver variability, sample heterogeneity, and benefit in tumors without PD-L1 expression, *etc*.), we must also include temporal variability. RT and CT both alter the tumor microenvironment and PD-L1 expression[66,67]. Therefore, perhaps the decision to administer durvalumab should be based on PD-L1 determination in the tumor sample after completion of concomitant CRT; however, this may be challenging in routine clinical practice (due to patient risks, lack of resources, *etc*.), and thus more reliable, stable biomarkers are needed.

Some ongoing studies, such as the aforementioned PACIFC-5 trial, will provide prospective data on the drug's efficacy in patients with varying expression level of PD-L1.

Therapeutic options in patients who progress on durvalumab

Despite the good results achieved with the PACIFIC regimen, a significant proportion of patients still develop disease progression. In fact, 40% of patients required additional treatment after completion of durvalumab therapy, but only 8% received immunotherapy. The efficacy and safety of immunotherapy retreatment in these patients is not known, nor do we know which patient subgroup would most benefit from such an approach (*e.g*., based on PD-L1 expression, longer progression-free interval, *etc*.). However, several studies are underway in an effort to answer these questions (NCT03526887), and the results of these trials could potentially be extrapolated to patients treated with durvalumab.

Another area of interest involves cases which progression is exclusively oligometastatic. A recent communication from the ASTRO 2019 congress reported findings from an exploratory analysis of data from the PACIFIC trial, demonstrating that most patients presented evidence of progression in only one or two sites after durvalumab therapy[67]. A retrospective, real-world study that analyzed data from patients treated with durvalumab after concomitant CRT found that nearly half had oligometastatic disease at the first progression[68]. These findings suggest that local ablative therapies could be applied in patients with oligometastatic progression.

CONCLUSION

Treatment with 12 mo of consolidation durvalumab therapy is the new standard of care in patients with unresectable stage III NSCLC who have received radical-intent concomitant CRT without subsequent progression. The addition of immunotherapy to the treatment of locally-advanced NSCLC represents a paradigm shift based on the findings from the PACIFIC trial showing that consolidation durvalumab (an anti-PD-L1 ICI) significantly improved both PFS and OS compared to placebo. Moreover, the toxicity profile was manageable, with no significant deterioration in quality of life. Even so, implementation of this strategy in routine clinical practice presents numerous challenges, requiring a multidisciplinary approach to decision-making to resolve important controversies such as patient selection, PD-L1 determination, the optimal timing of durvalumab initiation, and changes in clinical work flow practices.

Many questions remain unresolved. However, we will have greater clarity when results are published from the many trials currently underway to evaluate diverse strategies that combine immunotherapy with radical treatment in patients with locally-advanced NSCLC. It seems probable that the management of these patients will continue to evolve in the near future, especially those with stage IIIA disease. Nevertheless, it is evident that immunotherapy for the treatment of locally-advanced NSCLC is here to stay.

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ORIGINAL ARTICLE

Retrospective Study

Artificial intelligence in dentistry: Harnessing big data to predict oral cancer survival

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Institutional review board statement: This is not a human subject research study. Per the United States federal regulations (45 CFR 46, category 4), this study is deemed exempt and does not require review from Institutional Review Board since the data were deidentified and publicly available.

Informed consent statement: This

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Abstract

BACKGROUND

Oral cancer is the sixth most prevalent cancer worldwide. Public knowledge in oral cancer risk factors and survival is limited.

AIM

To come up with machine learning (ML) algorithms to predict the length of survival for individuals diagnosed with oral cancer, and to explore the most important factors that were responsible for shortening or lengthening oral cancer survival.

METHODS
We used the Surveillance, Epidemiology, and End Results database from the years 1975 to 2016 that consisted of a total of 257880 cases and 94 variables. Four ML techniques in the area of artificial intelligence were applied for model training and validation. Model accuracy was evaluated using mean absolute error (MAE), mean squared error (MSE), root mean squared error (RMSE), R^2 and adjusted R^2.

RESULTS
The most important factors predictive of oral cancer survival time were age at diagnosis, primary cancer site, tumor size and year of diagnosis. Year of diagnosis referred to the year when the tumor was first diagnosed, implying that individuals with tumors that were diagnosed in the modern era tend to have longer survival than those diagnosed in the past. The extreme gradient boosting ML algorithms showed the best performance, with the MAE equaled to 13.55, MSE 486.55 and RMSE 22.06.

CONCLUSION
Using artificial intelligence, we developed a tool that can be used for oral cancer survival prediction and for medical-decision making. The finding relating to the year of diagnosis represented an important new discovery in the literature. The results of this study have implications for cancer prevention and education for the public.

Key Words: Oral cancer survival; Machine learning; Artificial intelligence; Dental medicine; Public health; Surveillance, Epidemiology, and End Results; Quality of life

Core Tip: Oral cancer is the sixth most prevalent cancer worldwide. The goal of this study was to come up with machine learning algorithms to predict the length of oral cancer survival and to explore the most important factors that were responsible for it. Age at diagnosis, primary cancer site, tumor size and year of diagnosis were found to be the most important factors predictive of oral cancer survival. Year of diagnosis represents an important new discovery in the literature. Using artificial intelligence, we developed a tool that can be used for oral cancer survival prediction and for medical decision making.

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INTRODUCTION

To minimize the occurrence of oral cancer and improve one's quality of life, it is imperative to conduct screenings for early detection of head and neck carcinomas (HNC) on all high-risk dental patients. HNC, which is the umbrella term that includes oral cancer, are often located within the oral and nasal cavities, upper/lower pharynx, larynx, and the maxillary sinus[1-3]. Early screenings for identification of dysplastic tissue in the head and neck region are within the scope of care of the dental health providers. Oral cancer may be curable if detected early[4]. However, more than one-half of all oral and pharyngeal cancers in the United States were detected at late stages[4,5], thus the overall United States five-year survival rate for oral cancer was only 52 percent[6]. In 2012, there were 145000 deaths in the United States attributed to oral cancers[2]. Throughout the world, approximately 563826 diagnoses of oral cancer were reported, rendering it the sixth most common type of cancer in the world[7-10]. Although there is a downward trend in oral cancer incidence due to the rising awareness in the risks associated with tobacco use and alcohol consumption in the United States, a

is not a human subject research study. This study used secondary data that were already collected and were publicly available online. Therefore, signed informed consent form is not relevant.

Conflict-of-interest statement: The authors declare that there is no conflict of interest regarding this work.

Data sharing statement: The data supporting the findings of this study can be accessed at: https://seer.cancer.gov/.

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general lack of public awareness of the symptoms and other risk factors of oral cancer remains[4]. In 2016, a total of 48330 oral cavity and oropharyngeal cancer incidents were reported[11], and an increase of 225% of human papillomavirus-related oropharynx cancer was recorded[11]. Altogether, the need to address additional risk factors and increases in early screenings of oral cancers are key factors to improving cancer survival[12].

Among those with an oral cancer diagnosis, stage of tumor at time of diagnosis and treatment have been associated with survival[10,13]. Specifically, in a study by Sargeran *et al*[13] the survival rates were higher in patients with stages I or II cancer than those with stage III cancer at the time of the diagnosis. They further concluded that patients who had undergone radiotherapy alone had a lower survival rate than patients with a combination of surgery and radiotherapy, and that age and sex were not associated with survival. However, Warnakulasuriya *et al*[10] found that younger age was associated with higher 5-year relative survival rate.

Additionally, race has been associated with varying level of survival rates. A study using 1973-2002 data from Surveillance, Epidemiology, and End Results (SEER-18)[14] by Shiboski *et al*[15] revealed that the stage at diagnosis was related to 5-year relative survival rate among Whites and Blacks. The results indicated that Blacks had a significantly higher rate of cancer, mainly located on the tongue, with tumors larger than 4 cm in diameter at the time of diagnosis. Black men experienced lower 5-year relative survival rates compared to White men, especially for tongue cancer. Shiboski *et al*[15] explained that the differences in survivals across different races may be due to differences in access to, and utilization of healthcare services.

Due to the limited understanding of the disparities seen across cancer survivors and public knowledge on risk factors and symptoms, investigators in the past have suggested for primary care providers to put greater weight on initial screening and comprehensive soft-tissue exams[15]. Having a tool to accurately predict the survival time of oral cancer patients could help regulate the effects of psychological distress on physical and mental health outcomes after diagnosis. Medical decision-making tools based on fuzzy and soft set theories and artificial intelligence are effective for determination of cancer survival and enhancing disease awareness[16]. Awareness of the disease can lessen the burden of the disease on the survivors and their caretakers, and assist with medical and dental decision-making moving forward. The main purpose of this study was to apply artificial intelligence to build a model to predict the length of survival for those diagnosed with oral cancer as accurately and precisely as possible based on 40 plus years-worth of data representative of the United States' population. The secondary purpose was to explore the most important factors that were influencing the longevity of oral cancer survival.

MATERIALS AND METHODS

Data

Data from the SEER-18 database[14] were used to conduct this study. The SEER-18 database is a population-based registry that contains cancer-related data on individuals diagnosed with cancer from hospitals and laboratories in the United States[14]. The SEER-18 database does not contain data from Louisiana during hurricanes Katrina and Rita from July to December in 2005[14]. Institutional review board approval was not required for this study since the SEER-18 data were deidentified and publicly available online. The data that support the findings of this study are openly available at https://seer.cancer.gov/.

Oral cancer cases from the years 1975 to 2016 in the SEER-18 database[17] were identified by the International Classification of Diseases for Oncology, 3rd Edition (ICD-O-3) site codes (https://training.seer.cancer.gov/head-neck/abstract-code-stage/codes.html)[18]. Table 1 contains a list of all ICD-O-3 site codes that were identified for the oral cancer cases utilized in this study[18-20].

Analytical approach

The outcome of interest for this study was oral cancer survival time. Survival time represented the time of survival in months from the date of cancer diagnosis to the date of last contact[21,22].

Descriptive statistics of demographics and cancer characteristics (such as primary site, tumor size, laterality, *etc.*) were analyzed. Prediction of oral cancer survival time was modeled by using four machine learning (ML) algorithms: linear regression, decision tree, random forest, and extreme gradient boosting (XGBoost). ML is a

Table 1 Number of oral cancer cases from various anatomical sites

ICD-O-3 codes	Sites	Number of cases
C000	External upper lip	413
C001	External lower lip	2444
C002	External lip, NOS	92
C003	Mucosa of upper lip	104
C004	Mucosa of lower lip	567
C005	Mucosa of lip, NOS	29
C006	Commissure of lip	85
C008	Overlapping lesion of lip	46
C009	Lip, NOS (excludes skin of lip C44.0)	153
C019	Base of tongue, NOS	10840
C020	Dorsal surface of tongue, NOS	652
C021	Border of tongue	2632
C022	Ventral surface of tongue, NOS	1688
C023	Anterior 2/3 of tongue, NOS	2807
C024	Lingual tonsil	170
C028	Overlapping lesion of tongue	581
C029	Tongue, NOS	3050
C030	Upper gum	821
C031	Lower gum	1680
C039	Gum, NOS	210
C040	Anterior floor of mouth	1362
C041	Lateral floor of mouth	352
C048	Overlapping lesion of floor of mouth	136
C049	Floor of mouth, NOS	2284
C050	Hard palate	1155
C051	Soft palate, NOS (excludes nasopharyngeal surface of soft palate C11.3)	1301
C052	Uvula	180
C058	Overlapping lesion of palate	206
C059	Palate, NOS	154
C060	Cheek mucosa	1787
C061	Vestibule of mouth	134
C062	Retromolar area	1413
C068	Overlapping lesion of other and unspecified parts of mouth	142
C069	Mouth, NOS	487
C079	Parotid gland	7111
C080	Submandibular gland	1149
C081	Sublingual gland	94
C088	Overlapping lesion of major salivary glands	6
C089	Major salivary gland, NOS (excludes minor salivary gland, NOS C06.9)	287
C090	Tonsillar fossa	1735
C091	Tonsillar pillar	888

C098	Overlapping lesion of tonsil	109
C099	Tonsil, NOS (excludes lingual tonsil C02.4 and pharyngeal tonsil C11.1)	9521
C100	Vallecula	282
C101	Anterior surface of epiglottis	88
C102	Lateral wall of oropharynx	184
C103	Posterior wall of oropharynx	246
C104	Branchial cleft (site of neoplasm)	37
C108	Overlapping lesion of oropharynx	277
C109	Oropharynx, NOS	940
C129	Pyriform sinus	1707
C130	Postcricoid region	78
C131	Hypopharyngeal aspect of aryepiglottic fold, NOS (excludes laryngeal aspect of aryepiglottic fold C32.1)	214
C132	Posterior wall of hypopharynx	250
C138	Overlapping lesion of hypopharynx	113
C139	Hypopharynx, NOS	816
C739	Thyroid gland	111425

ICD-O-3 codes: International Classification of Diseases for Oncology, 3rd ed; NOS: Not otherwise specified.

computer algorithm-based method that can efficiently detect relationships between variables with unrecognizable trends in large and complex data. The process takes into account historical trends to come up with models in predicting outcome of interest (*e.g.*, oral cancer survival time), and then validates the models with actual or current data. The performance of the various models from the validation process will be compared, and the more parsimonious model with better performance is generally the preferred model. The ML techniques included in this study were chosen due to their ability to prevent over-fitting, being commonly used in similar studies, and their ease of interpretation in medical settings. To compare the different techniques, model accuracy was evaluated using mean absolute error (MAE)[1,13], mean squared error (MSE), root mean squared error (RMSE), R^2 and adjusted R^2. All analyses were conducted using Python 3.7.4 (Python Software Foundation).

There was a total of 257880 oral cancer cases and 94 variables (*i.e.*, features) in the dataset. Cases with missing data on the outcome variable (*i.e.*, oral cancer survival time) were dropped, and responses that were marked as not applicable were excluded. All variables with more than 40% of missing values were also excluded. Further data processing was conducted to remove null features, constant features (*i.e.*, features with same values for the outcome), quasi-constant features (*i.e.*, features with variance less than 0.01), and highly correlated features (*i.e.*, features with correlation higher than 0.9). These features were removed prior to data analysis as they would not contribute to the prediction of outcome and can often cause errors in the prediction. Outliers were detected by plotting distributions of each variable and they were replaced by mean, mode, and quantile as appropriate. Features with more than 90% the values that were the same were dropped.

To avoid the impracticality of including too many variables, further feature selection was performed using random forest. We aimed to narrow down the variables as much as possible without losing prediction accuracy. The random forest model showed that many features are of little importance (Figure 1). We dropped 7 features that were of less importance in terms of their importance scores, and a step backward feature selection method with random forest was then applied to select the best number of features. The cross-validation scores were then plotted (Figure 2) and the most important 10 features were kept to create a parsimony model. The cross-validation scores did not change much even after deleting the less significant features. The selected 10 features were: Year of diagnosis; primary site; age at diagnosis; CS tumor size; CS extension; CS lymph nodes eval; RX Summ-surg prim site; derived AJCC stage group; site recode ICD-O-3/WHO 2008; and month of diagnosis.

The final dataset used for model prediction from linear regression, decision tree,

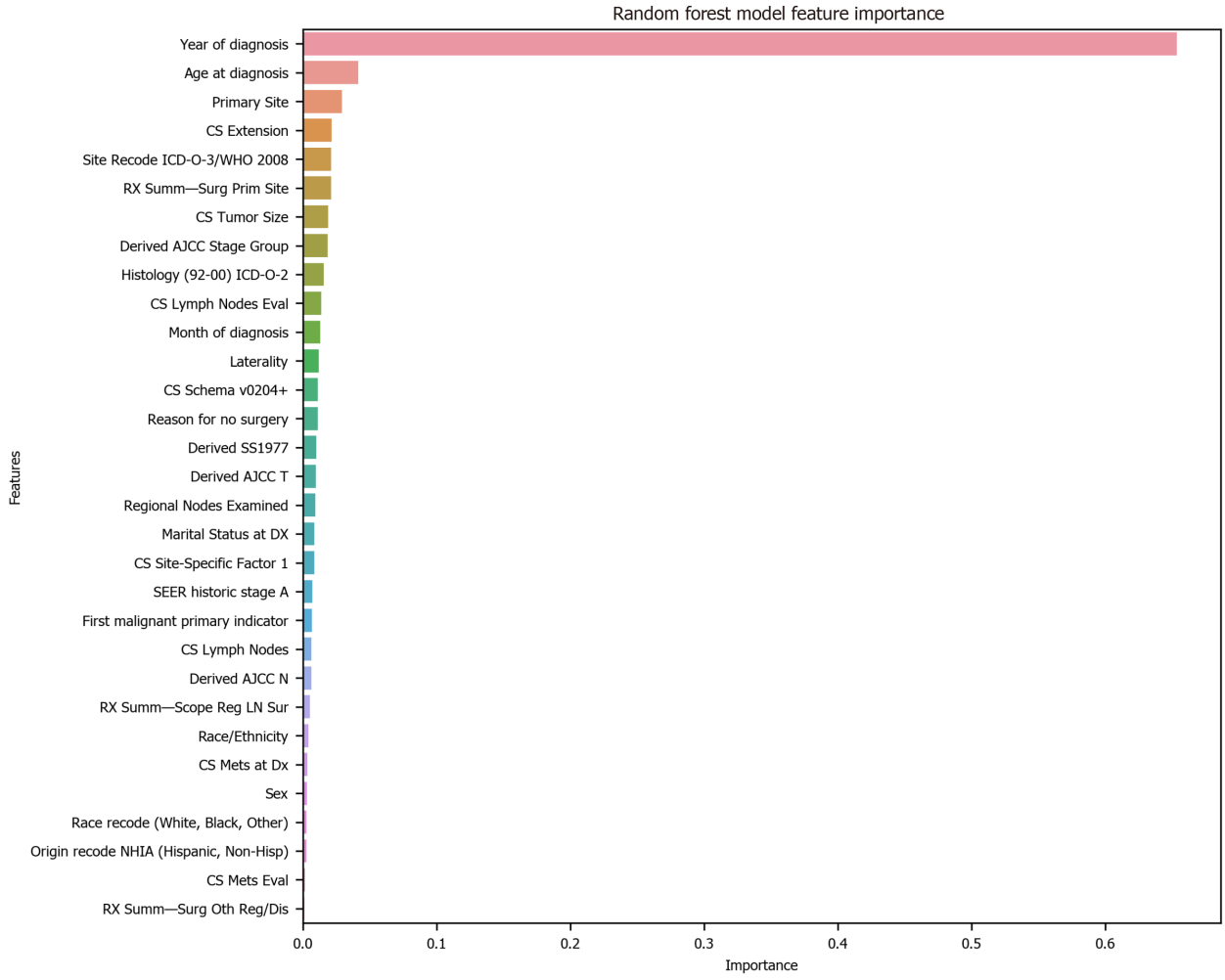


Figure 1 Feature selection using random forest. CS: Coding system; ICD-O-3: International Classification of Diseases for Oncology, 3rd ed; WHO: World Health Organization; AJCC: American Joint Committee on Cancer; SEER: Surveillance, Epidemiology, and End Results; LN: Lymph node.

random forest, and XGBoost had an effective sample size of 177714 cases with a total of 10 variables. Most of the values were categorized and given numerical code values. Table 2 lists all of the variables. Data were randomly split into training set and testing set. The training set contained 75% of the data and were used to build models. The testing set contained 25% of the data and were used to validate the models built from the training data. Detailed model parameter tuning set up is available upon request from the authors.

RESULTS

There was a total of 177714 oral cancer cases included in the study, of which 63111 were oropharyngeal cancer cases and 114603 were laryngeal cancer cases. The nasopharyngeal cancer cases did not make it to the final sample since there was very few of these cases and all of them had a large number of missing values. Oropharynx cancer included anatomical positions at the base of tongue, lingual tonsil, soft palate, uvula, tonsil, orpharynx, Waldeyer ring, and histology sites[23]. Laryngeal cancer included areas at the larynx, which comprises of the epiglottis, supraglottis, vocal cord, glottis, and subglottis[24]. The sample consisted of 40.62% (n = 72179) males. The average age at diagnosis was 54.6 years old (range: 0-109) (Figure 3). Nearly 40% of the sample were 60 years or older at the time of oral cancer diagnosis (Table 3).

Among the 10 features, several of them showed strong linear relation with survival time (Figure 4). Hence a linear regression model was used to predict outcome. The feature importance can be visualized in Figure 4 showing year of diagnosis as the most important variable. The performance of linear regression was MSE = 647.49, RMSE = 25.45, MAE = 18.21, R^2 = 0.620 and adjusted R^2 = 0.620 (Table 4).

Table 2 List of all 10 variables included in the final machine learning model building and validation

Variables	Variable description
Age at diagnosis	This data item represents the age of the patient at diagnosis for this cancer. The code is three digits and represents the patient's actual age in years
Year of diagnosis	The year of diagnosis is the year the tumor was first diagnosed by a recognized medical practitioner, whether clinically or microscopically confirmed
Month of diagnosis	The month of diagnosis is the month the tumor was first diagnosed by a recognized medical practitioner, whether clinically or microscopically confirmed
Primary site	This data item identifies the site in which the primary tumor originated. See the International Classification of Diseases for Oncology, 3rd Edition (ICD-O-3)[18] for topography codes. The decimal point is eliminated
CS tumor size	Information on tumor size. Available for 2004-2015 diagnosis years. Earlier cases may be converted and new codes added which weren't available for use prior to the current version of CS. For more information, see http://seer.cancer.gov/seerstat/variables/seer/ajcc-stage[19]
CS extension	Information on extension of the tumor. Available for 2004-2015 diagnosis years. Earlier cases may be converted and new codes added which weren't available for use prior to the current version of CS. For more information, see http://seer.cancer.gov/seerstat/variables/seer/ajcc-stage[19]
CS lymph nodes eval	Available for 2004-2015, but not required for the entire timeframe. Will be blank in cases not collected. For more information, see http://seer.cancer.gov/seerstat/variables/seer/ajcc-stage[19]
Derived AJCC stage group	This is the AJCC "Stage Group" component that is derived from CS detailed site-specific codes, using the CS algorithm, effective with 2004-2015 diagnosis years. See the CS site-specific schema for details (http://seer.cancer.gov/seerstat/variables/seer/ajcc-stage)[19]
RX Summ-surg prim site	Surgery of primary site describes a surgical procedure that removes and/or destroys tissue of the primary site performed as part of the initial work-up or first course of therapy
Site recode ICD-O-3/WHO 2008	A recode based on primary site and ICD-O-3 Histology in order to make analyses of site/histology groups easier. For example, the lymphomas are excluded from stomach and Kaposi and mesothelioma are separate categories based on histology. For more information, see http://seer.cancer.gov/siterecode/icdo3_dwhoheme/index.html[20]

CS: Coding System; AJCC: American Joint Committee on Cancer; ICD-O-3: International Classification of Diseases for Oncology, 3rd ed; WHO: World Health Organization.

Decision tree regression, a ML method, was used to determine the top features (*i.e.*, variables) that were predictive of oral cancer survival time. Relative variable importance scores were computed to identify the top predictors. The usage of the decision tree regression was ideal as it doesn't require linear relationship between features and target variable. Year of diagnosis was found as the most important variable (Figure 5). The performance of the decision tree was MSE = 538.30, RMSE = 23.20, MAE[1] = 14.45, R^2 = 0.681 and adjusted R^2 = 0.681 (Table 4).

Among the 10 features, several of them showed strong linear relation with survival time (Figure 4). Hence a linear regression model was used to predict outcome. The feature importance can be visualized in Figure 4 showing year of diagnosis as the most important variable. The performance of linear regression was MSE = 647.49, RMSE = 25.45, MAE = 18.21, R^2 = 0.620 and adjusted R^2 = 0.620 (Table 4).

Random forest method was also conducted to develop predictive model. It was appropriate for data with one strong predictor and some moderate predictors. The feature importance for random forest is shown in Figure 4 with year of diagnosis as the most important variable. The performance of the random forest was MSE = 489.58, RMSE = 22.13, MAE = 13.63, R^2 = 0.709 and adjusted R^2 = 0.709 (Table 4).

Finally, the XGBoost model was used. The performance of the XGBoost was MSE = 486.55, RMSE = 22.06, MAE = 13.55, R^2 = 0.711 and adjusted R^2 = 0.711 (Table 4). The feature importance for the XBoost model is presented in Figure 4 showing primary cancer site and year of diagnosis as the top two most important variables for prediction of oral cancer survival. Figure 6 presents a comparison of the prediction of oral cancer survival time from all models against the actual survival time. All model predictions were very similar and close to the actual outcomes. When the survival time was between 40 mo and 60 mo, the predictions were on target with the actual survival time. When it was under 40 mo, the predicted survival time for all models were slightly higher than the actual survival time. However, when it was over 60 mo, the predicted survival time for all models were slightly lower than the actual survival.

Table 3 Demographic characteristics of the sample (*n* = 177714)

Variable	Mean	SD	Median	*n*	%
Survival months/mo	60.35	40.98	54.00		
Age at diagnosis/yr	54.62	16.10	55.00		
Tumor size/(ID, cm)	22.56	21.74	19.00		
Marital status					
Single				35688	20.08
Married				110480	62.17
Separated				1746	0.98
Divorced				16401	9.23
Widowed				13055	7.35
Unmarried or domestic partner				344	0.19
Sex					
Male				72179	40.62
Female				105535	59.38
Race					
White				148556	83.60
Black				16051	9.03
Other				13107	7.38

SD: Standard deviation; ID: Diameter.

Table 4 Machine learning model performance

Performance indicators	Linear regression	Decision tree	Random forest	XGBoost
MSE	647.49	538.30	489.58	486.55
RMSE	25.45	23.20	22.13	22.06
MAE	18.21	14.45	13.63	13.55
R^2 score	0.620	0.681	0.709	0.711
Adjusted R^2 score	0.620	0.681	0.709	0.711

XGBoost: Extreme gradient boosting; MSE: Mean squared error; RMSE: Root mean squared error; MAE: Mean absolute error.

DISCUSSION

The goal of this study was two-fold: (1) To build a ML model predictive of the length of survival for those diagnosed with oral cancer, and (2) To establish the most important factors that predict oral cancer survival. Our results showed that XGBoost was the best model in terms of accuracy. XGBoost's performance exceeded all other ML methods, with linear regression's performance slightly trailing behind all models. The average length of survival for all patients was 60.35 mo. Furthermore, age at diagnosis, primary cancer site, tumor size and year of diagnosis were the most important factors related to oral cancer survival. Year of diagnosis was consistently ranking as the top feature across all models. Year of diagnosis was not the number of years nor the amount of time since the tumor was initially diagnosed. Rather, year of diagnosis referred to the year when the tumor was first diagnosed, implying that individuals with tumors that were diagnosed in the modern era tend to have longer survival than those diagnosed in the past.

To our knowledge, this study is the first of its kind to use ML techniques to predict length of survival for those diagnosed with oral cancer. Previous research is consistent with some of our findings. Tumor size, specifically thickness among other tumor size

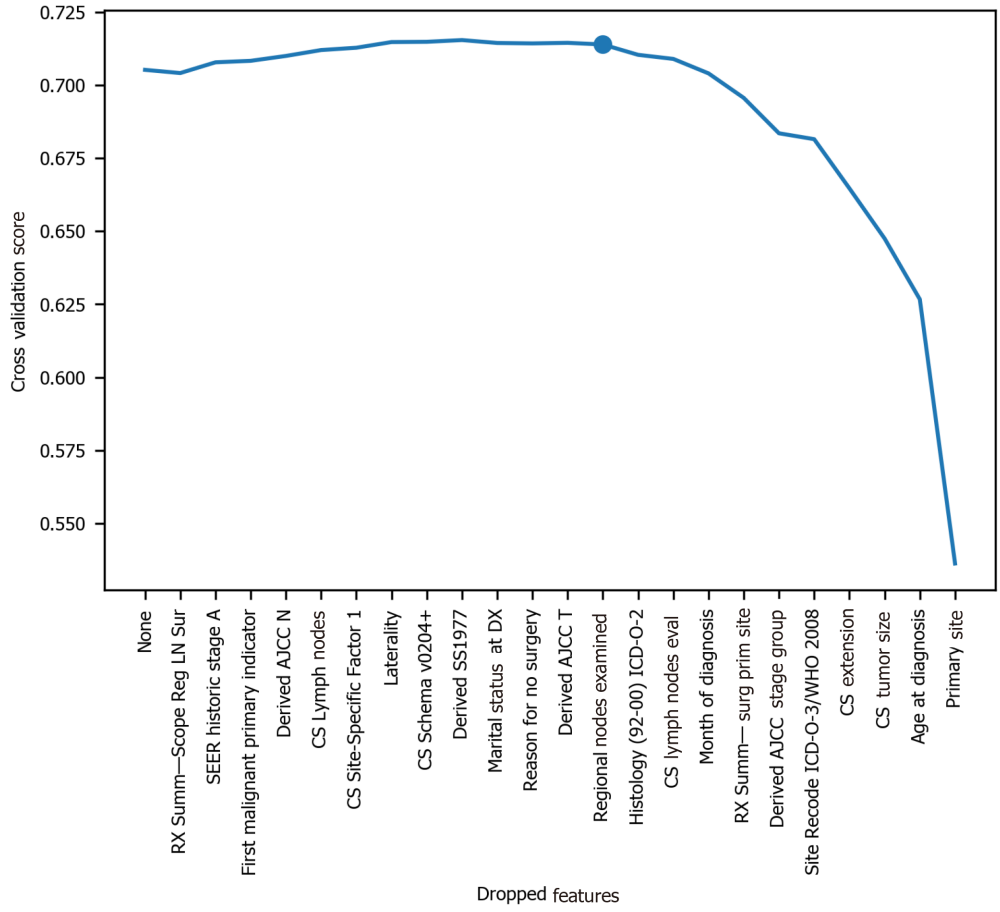


Figure 2 Cross-validation score change for selecting optimal number of features. LN: Lymph node; SEER: Surveillance, Epidemiology, and End Results; AJCC: American Joint Committee on Cancer; CS: Coding system; ICD-O-3: International Classification of Diseases for Oncology, 3rd ed; WHO: World Health Organization.

parameters, has been found to be a significant predictor of oral tongue carcinoma survival[25,26]. Younger patients with oral cavity squamous cell cancer[27] and squamous cell carcinoma of the oral tongue[28] have been found to have a higher survival rate in the past which is also consistent with our findings. For cases of squamous cell carcinoma of the oral tongue, a ten-year increase in age was associated with an 18% increase in risk of death[27,28]. However, year of diagnosis was a unique and novel predictive factor that has not been reported in the literature. Considering that our study included 40 plus years-worth of data and incorporated ML for precise prediction, this perhaps makes it possible for discovering new knowledge. It is possible that more recent year of diagnosis leads to the better survival outcomes due to improved oral cancer treatments and public awareness.

This study also revealed some conflicting findings that need further exploration. Although race and ethnicity have been identified as predictors to oral cancer survival in past literature[15], our study using recent data showed low importance of these features, so race and ethnicity were eventually dropped from the model. Given that our study included 40 plus year-worth of data and consisted of recent data, we may see that race and ethnicity are not associated with oral cancer survival over time. Improvements in access to, and utilization of healthcare services among race could also be reasons leading to no or low racial disparities in oral care in the 21st century. Additional large-scale studies using recent data are needed to evaluate these findings.

A primary limitation of this study was that the data did not include psychological factors that could explain survivors' quality of life. In a future study, we can explore other databases and incorporate surveys to explain the psychological state of oral cancer survivors and overall perspective on the disease. Over 50% of diagnosed oral cancer cases still remain a lethal disease annually[10], early detection and accessibility to regular head and neck examination is key.

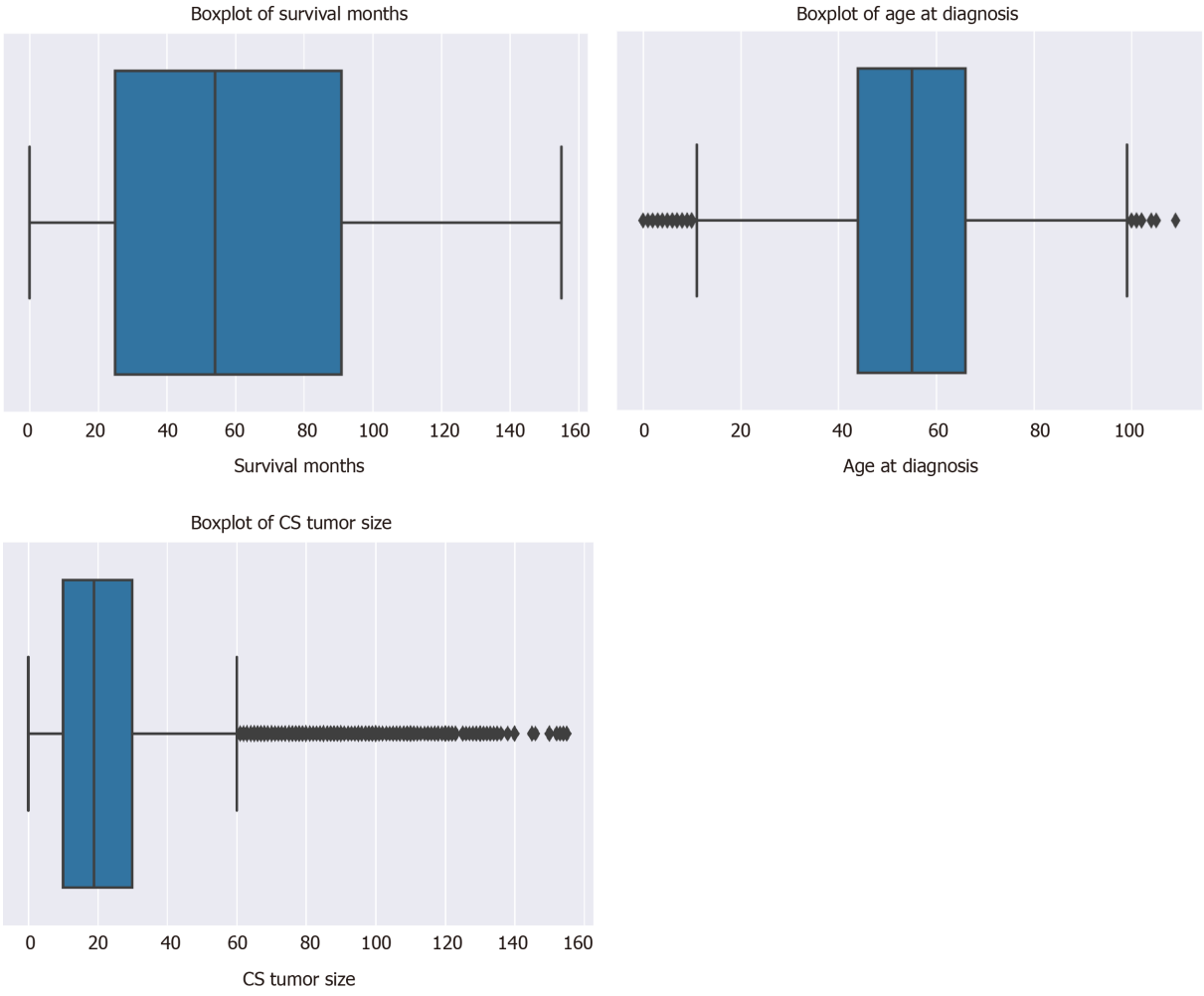
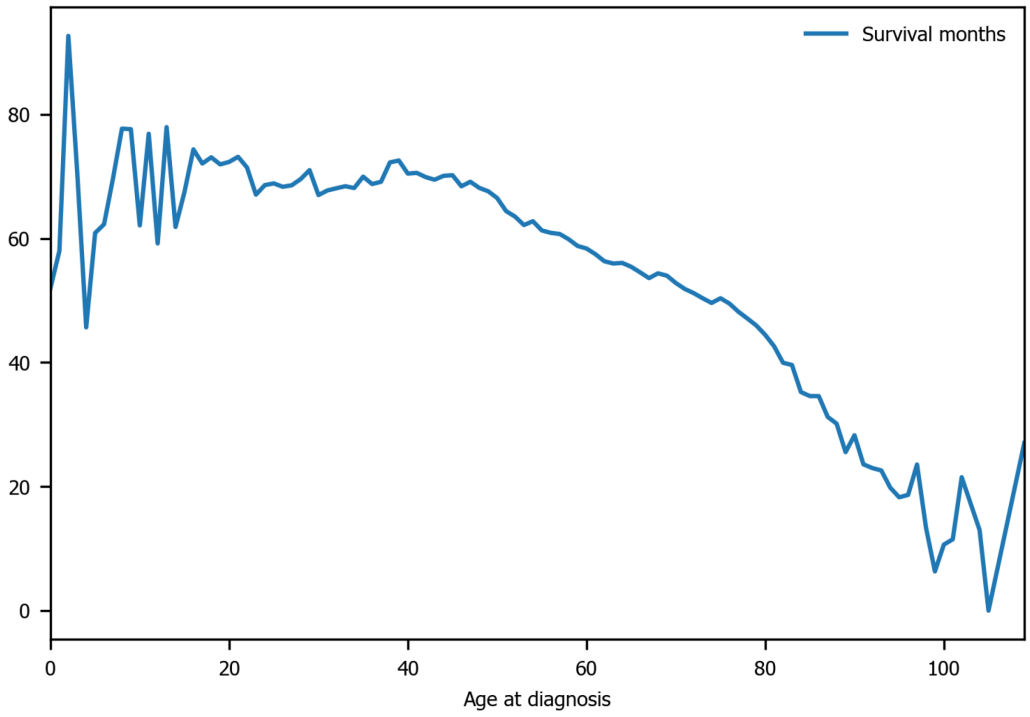
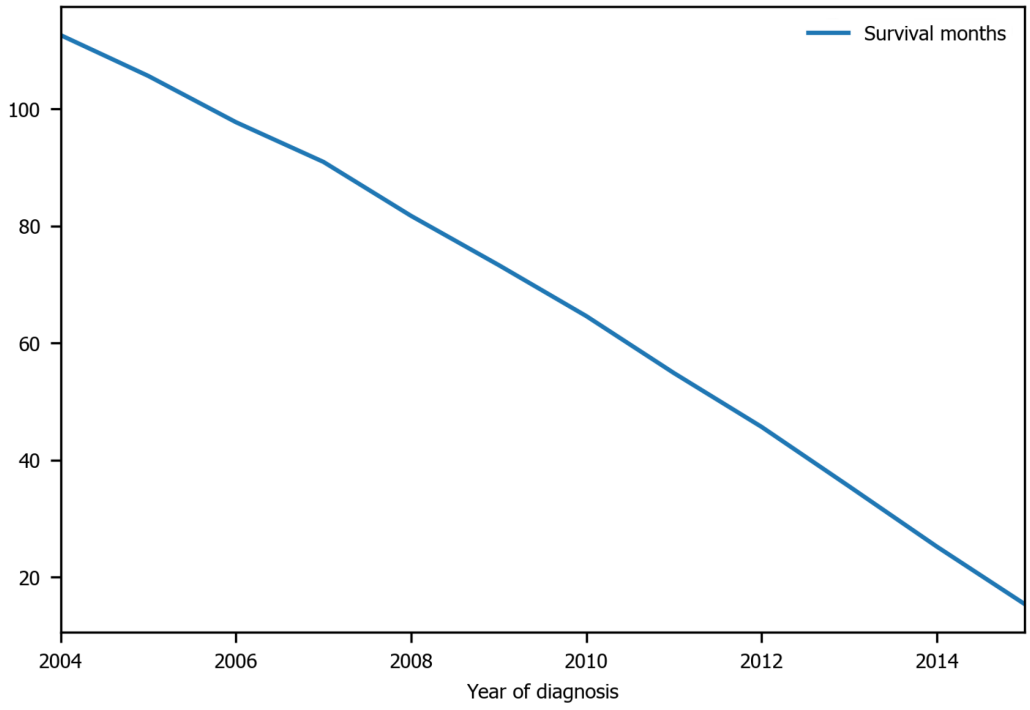


Figure 3 Boxplots of sample characteristics. CS: Coding system.

CONCLUSION

This study is particularly important and appropriate for the field of dentistry as the prediction of oral cancer survival can assist dentists, patients and caregivers in disease management and treatment plan development. Identifying oral cancer and gaining a more in depth understanding of the length of survival for those diagnosed with oral cancer and establishing important factors that predict oral cancer survival will better equip health care providers on how to best manage such diagnoses. This study serves as a steppingstone for future exploration using ML and artificial intelligence to uncover the full potential for the management of oral cancers and to reduce healthcare disparities around the globe.


Survival months
80
60
40
20
0
0
20
40
60
80
100
Age at diagnosis


Survival months
100
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40
20
2004
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Year of diagnosis

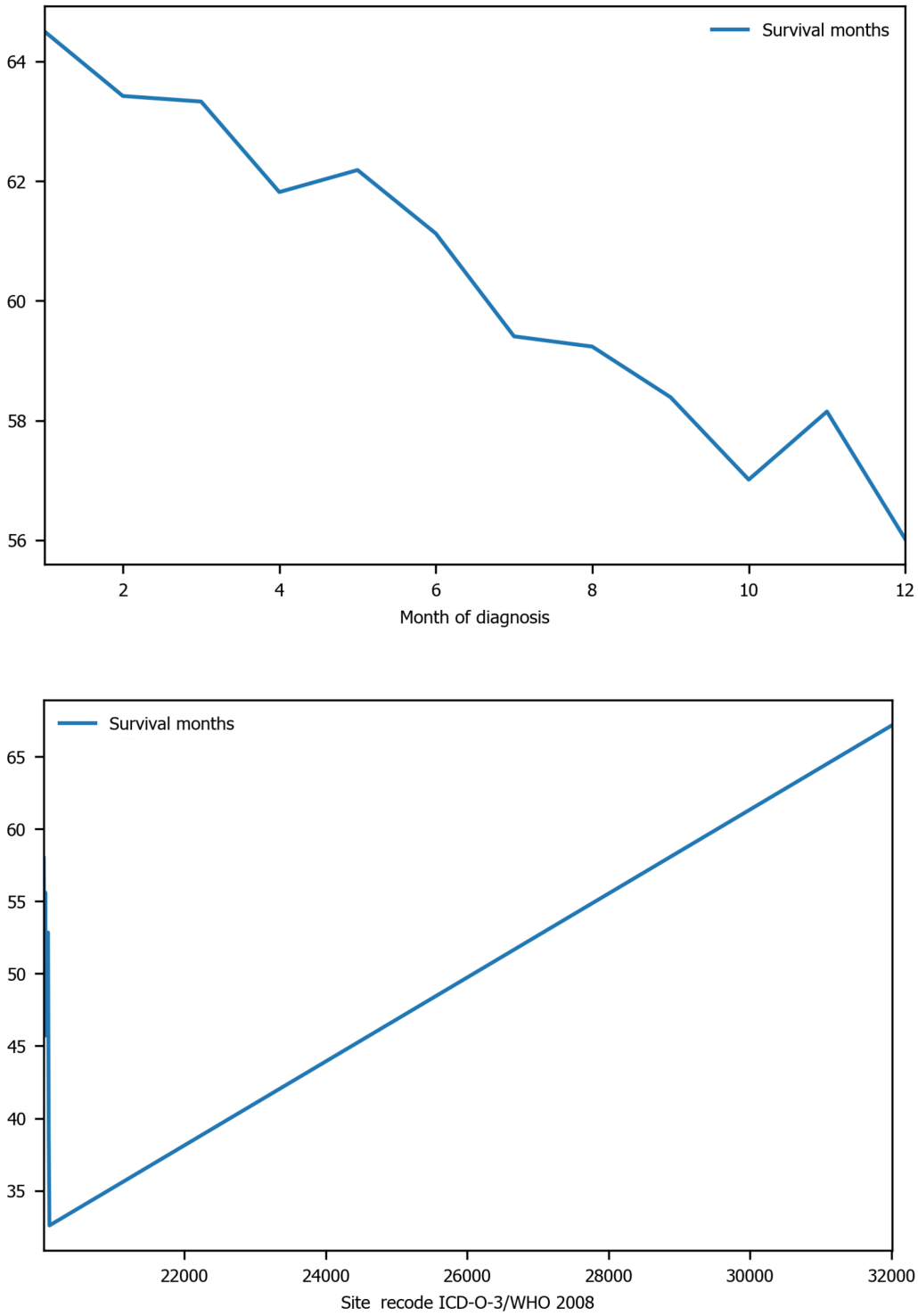
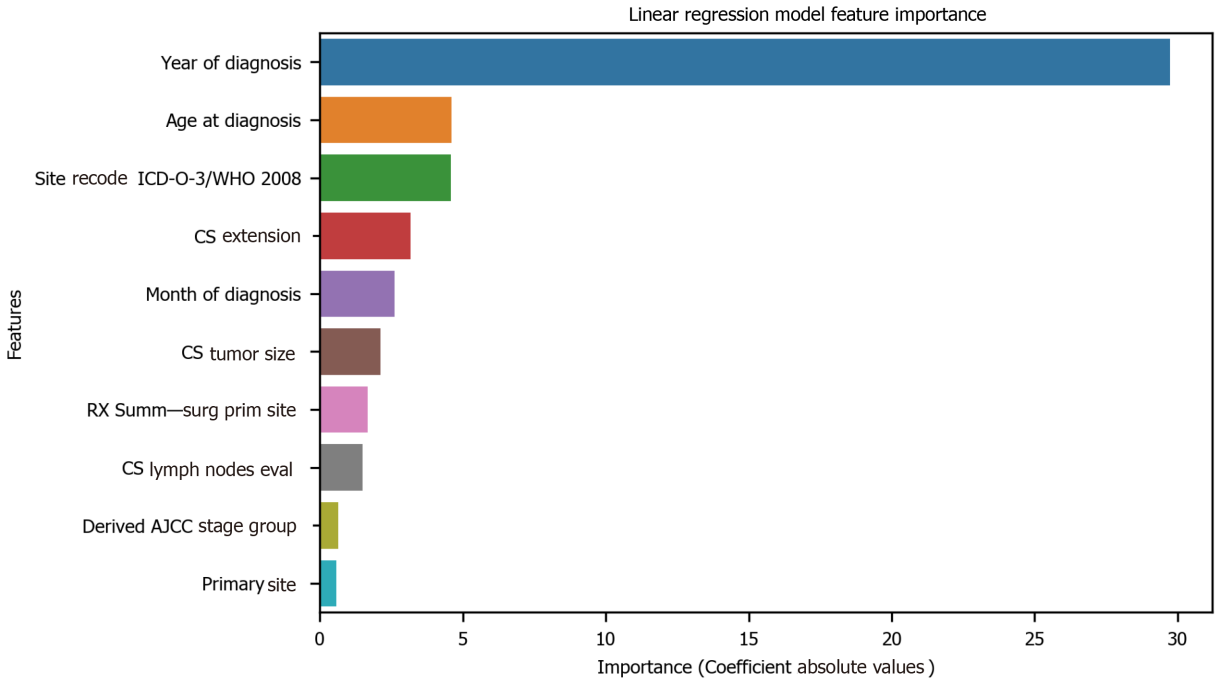
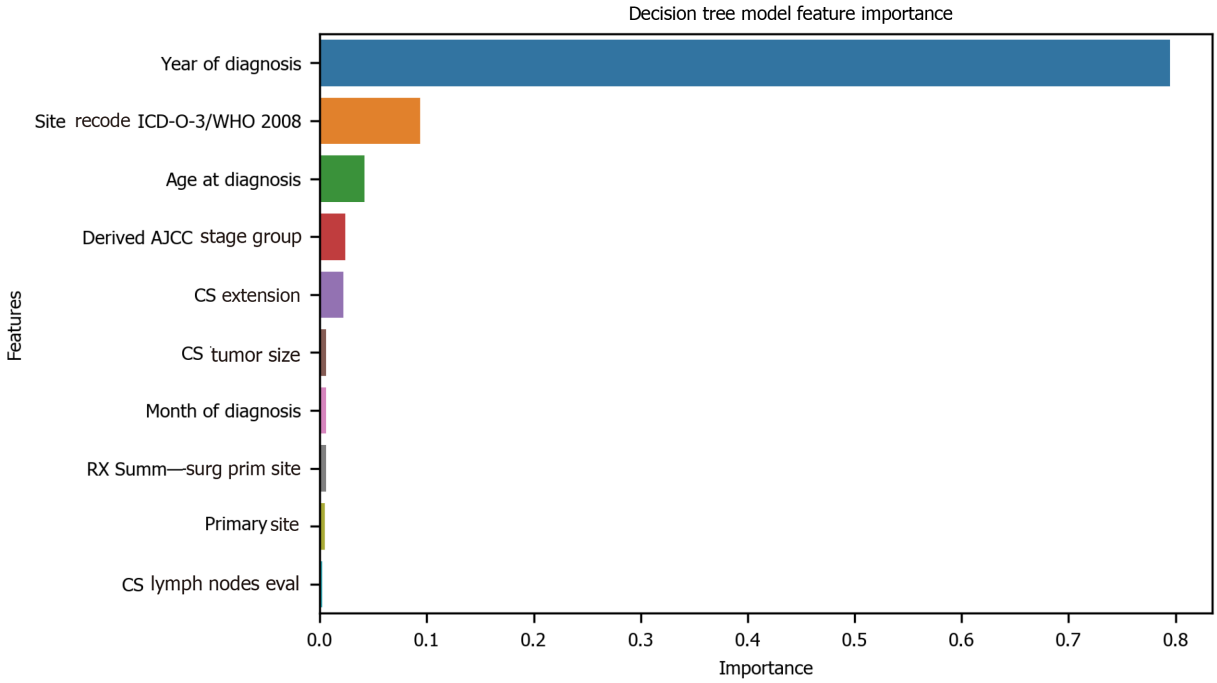


Figure 4 Survival months shows strong linear relation with several variables: Age of diagnosis, year of diagnosis, month of diagnosis, and site recode ICD-O-3/WHO 2008. ICD-O-3: International Classification of Diseases for Oncology, 3rd ed; WHO: World Health Organization.


Linear regression model feature importance
Year of diagnosis
Age at diagnosis
Site recode ICD-O-3/WHO 2008
CS extension
Month of diagnosis
CS tumor size
RX Summ—surg prim site
CS lymph nodes eval
Derived AJCC stage group
Primary site
Features
0
5
10
15
20
25
30
Importance (Coefficient absolute values)


Decision tree model feature importance
Year of diagnosis
Site recode ICD-O-3/WHO 2008
Age at diagnosis
Derived AJCC stage group
CS extension
CS tumor size
Month of diagnosis
RX Summ—surg prim site
Primary site
CS lymph nodes eval
Features
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Importance

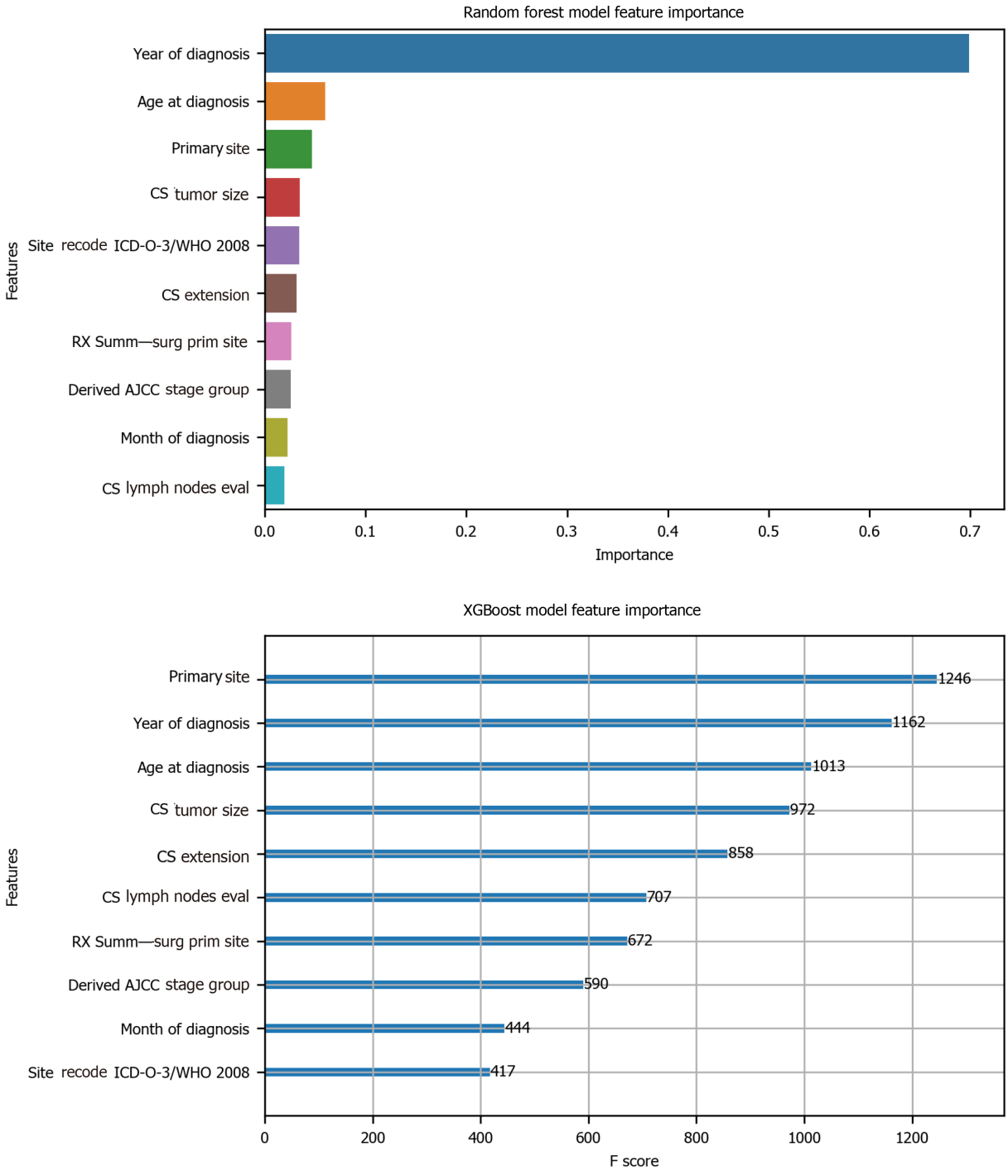


Figure 5 Machine learning model feature importance. ICD-O-3: International Classification of Diseases for Oncology, 3rd ed; WHO: World Health Organization; CS: Coding system; AJCC: American Joint Committee on Cancer.

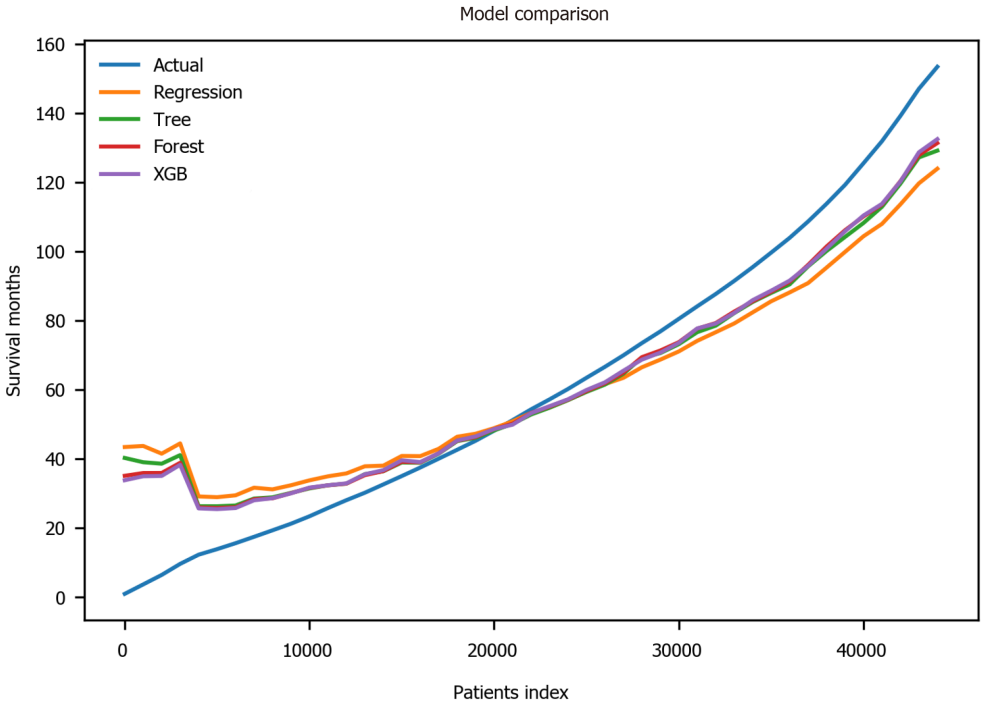


Figure 6 Prediction comparison among different models. Patient index refers to the rank after sorting by survival months. Actual: The actual survival outcome; XGB: Extreme gradient boosting.

ARTICLE HIGHLIGHTS

Research background

Oral cancer is highly prevalent in the world, yet there is a limited understanding of oral cancer risk factors and survival.

Research motivation

To increase one's quality of life, it is important to be able to predict oral cancer survival.

Research objectives

The objectives of this study were to build an accurate model to precisely predict the length of oral cancer survival and to explore the most important factors that determine the longevity of oral cancer survivors.

Research methods

Oral cancer data were obtained from the years 1975 to 2016 in the Surveillance, Epidemiology, and End Results database. Methods from the field of artificial intelligence were applied to build and validate prediction models from 40+ years of oral cancer data representative of the United States' population.

Research results

Age at diagnosis, primary cancer site, tumor size and year of diagnosis were the most important factors related to oral cancer survival. Individuals with tumors that were diagnosed in the modern era tend to have longer survival than those diagnosed in the past, which was a novel finding that had not been reported in the literature.

Research conclusions

Machine learning algorithms were developed this study to predict the length of oral cancer survival that can be readily deployed to clinical settings.

Research perspectives

This study was the first of its kind to use methods from artificial intelligence to examine the length of survival for individuals diagnosed with oral cancer. The outcome of this study has the potential to reduce healthcare disparities and improve

the quality of life for oral cancer survivors and their friends and families around the world.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Prospective Study

Assessment of breast cancer immunohistochemistry and tumor characteristics in Nigeria

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Author contributions: Aliyu UM drafted the methodology and discussion; Musa AA assisted with data analysis and interpretation of the result; Aliyu UM and Musa AA performed the writing and editing for the original manuscript.

Institutional review board statement: This study was reviewed and approved by the Research Ethics Committee. The research topic is in line with the rule and regulation of the institution.

Conflict-of-interest statement: The authors of this manuscript have no conflicts of interest to disclose.

Data sharing statement: There are no additional data available.

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Abstract

BACKGROUND

Female breast cancer is the leading type of cancer worldwide with an incidence of approximately 2.1 million in 2018. Hormone receptor status plays a vital role in its management.

AIM

To determine the molecular expression pattern of biomarkers in breast cancer and their correlation with tumor variables.

METHODS

This prospective study was designed to analyze expression patterns of estrogen receptor(ER), progesterone receptor (PR) and human epidermal growth factor receptor(HER2/neu) in breast cancer patients. The dataset has been taken from the Department of Radiotherapy and Oncology of Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria from 1 January 2015 to 2 December 2019. The dataset had 259 records and 7 attributes. SPSS version 23.0 for statistical analysis was used. The data analyzed demographic and other clinicopathological characteristics as categorical variables. The mean and standard deviation were determined for the quantitative variable.

RESULTS

A total of 259 breast cancer cases were included in the study. The mean age was 48.3 ± 11.0, with an age range of 26-80 years and a median age of 46 years. The morphological categories were invasive ductal carcinoma 258 (99.6%) and invasive lobular carcinoma 1 (0.4%). ER, positivity increased in 73 patients (50%) under the age of 50 years, as well as PR positivity increased in 34 patients (23.6%) under the age of 50 years. HER/2neupositivity decreased in 8 patients (5.6%)

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under the age of 50 years. Hormonal receptors were statistically significant with clinicopathological characteristics ($P < 0.05$).

CONCLUSION
Our study showed that ER, PR and HER2/neuexpression had a strong correlation with age, tumor grade, tumor size and lymph node status. Hence, hormone receptor assessment is highly recommended because of its significance in clinical management and prognostication.

Key Words: Breast cancer; Hormonal receptors; Sokoto state; Tertiary Health Institution; Northwestern; Nigeria

Core Tip: Breast cancer is the most common malignancy in Nigeria as it is in the whole world. Assessment of immunohistochemical profile and its tumor characteristic is paramount to the management of this disease entity. The paucity of literature on breast cancer biomarkers in sub-Saharan Africa (*e.g.*, Nigeria) has contributed to the increase in morbidity and mortality recorded in the region.

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INTRODUCTION

Female breast cancer is the most common malignancy worldwide, with over two million cases diagnosed in 2018[1]. Prognostic indicators are features that are unrelated to adjuvant therapy that determines the outcome of disease, while predictive indicators are related to response to therapy[2]. The treatment and outcome of this disease entity vary depending on the biological characteristics. Hence, modalities for treatment employ those features to individualized breast cancer therapy[3]. These characteristics can also determine the prognosis and outcome[2]. In the last three decades, tremendous progress has been made in the biological characterization of breast cancers with the advent of immunohistochemistry and immunophenotyping. Various biomarkers such as hormone receptors, vascular endothelial growth factors, epithermal growth factor, tumor suppressor genes, multidrug resistant genes and adhesion molecules have been identified[2,4].

Currently, determination of estrogen receptor (ER), progesterone receptor (PR) and human epidermal receptor growth factor 2 (HER2/neu) receptor is routine in the diagnosis of breast cancer[5,6]. The International Breast Cancer Study Group classified breast cancer into three[6,7] categories based on the proportion of cells that are receptor positive [responsive (10%), response uncertain (1%-9%) and nonresponsive (0%)]. At least 1% positivity is necessary for commencement of hormone therapy. Molecular assays give an insight into the current status of the tumor, which often differs from the primary when it metastasizes. A comparative study involving 658 and 418 of ER/PR samples showed a dissonant rate of 29% and 27%, respectively, for primary and metastatic tumors[3,8].

ER is a biomarker found in over 65% of invasive breast cancer and contributes significantly to its pathobiology. ER positivity makes it responsive to hormonal therapy, resulting in a more favorable outcome. PR, like ER, is also a transcription factor, which is largely controlled by ER and to a lesser extent by growth factors. About 55% to 65% of invasive breast cancers show PR positivity. PR commonly coexists with ER. Both receptors require at least 1% nuclear staining to be considered positive according to the American Society of Clinical Oncology/College of American Pathologists guidelines[11,12]. Allred scoring and H-scoring are two other commonly used systems for ER and PR evaluations[9-11].

HER2/neu has the potential of enhancing proliferation and survival of tumor cells.

Hence its overexpression, which occurs in about 12%-20% of inflammatory breast cancer, results in a more aggressive growth and poor response to treatment. According to the American Society of Clinical Oncology/College of American Pathologists guidelines, membrane staining of 10% or more is considered positive and an indication of a favorable response to anti-HER2/neu therapy[11,13]. HER2/neu can be evaluated either by immunohistochemistry or fluorescence in situ hybridization. Although there are many ways to test for this receptor, the above two are the only approved methods[14-16].

This study aimed to determine the molecular expression of biomarkers in breast cancer in Sokoto, Nigeria and their correlation with tumor variables.

MATERIALS AND METHODS

This prospective study was designed to analyze expression patterns of ER, PR and HER2/neu in breast cancer patients. The dataset was taken from the Department of Radiotherapy and Oncology of Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria from 1 January 2015 to 2 December 2019. The dataset had 259 records and 7 attributes. The data analyzed demographic and other clinicopathological characteristics as categorical variables. The mean and standard deviation were determined for the quantitative variable. All variables were coded as binary dummy variables. For example tumor site (right breast = 1, left breast = 2). Patients were grouped according to age < 50 years and ≥ 50 years. Histological grading stratification of tumor size was performed in three groups (< 2 cm, 2-5 cm and > 5 cm), and lymph nodes were grouped as 1-3 and ≥ 4 based on tumor node metastasis staging for breast carcinoma.

Data were presented as charts, and frequency distribution was generated for all categorical variables. Descriptive and inferential statistics [chi-squared, Fisher exact test, bi-variate correlation (Kendall's test) and multivariate logistic regression analysis] were used to explore association, the strength of the association and odds ratio (OR) or relative risk between clinicopathological variables and biomarkers expressed (ER, PR and HER2/neu). $P \leq 0.05$ was considered statistically significant. SPSS version 23.0 (Chicago, IL, United States) was used.

RESULTS

Two hundred and fifty-nine subjects that fulfilled the inclusion criteria were recruited into the study. The mean age was 48.3 ± 11.0. The ages ranged between 26 and 80 years, and the median age was 46 years. Most of the patients 144 (55.6%) were less than 50 years at diagnosis (Figure 1). The left side was the predominant site of breast cancer 151 (58.3%) (Figure 2). The morphological categories were invasive ductal carcinoma (258 patients, 99.6%) and invasive lobular carcinoma (1 patient, 0.4%). Sixty-two (23.9%) cases were grade I, 131 (50.6%) were grade II and 66 (25.5%) were grade III. Tumor sizes were stratified into three categories: 62 patients (23.9%) were < 2.0 cm, 131 patients (50.6%) were 2-5 cm and 66 patients (25.5%) were > 5.0 cm (Table 1).

ER positivity increased in 73 patients (50%) under the age of 50 years, and PR positivity increased in 34 patients (23.6%) under the age of 50 years. HER2/neu positivity decreased in 8 patients (5.6%) under the age of 50 years ($P < 0.01$). ER positivity was found in 35 patients (56.5%), 55 patients (42.0%) and 23 patients (34.8%) with grade I, II and III carcinomas, respectively. PR positivity was found in 3 patients (3.2%), 29 patients (22.1%) and 10 patients (15.2%) with grade I, II and III carcinomas, respectively. HER2/neu positivity was found 3 patients (4.8%), 13 patients (9.9%) and 7 patients(10.6%) with grade I, II and III carcinomas, respectively. ER and PR positivity were much less in high-grade tumors than in those with intermediate grade (ER 34.8% *vs* 42%; PR 15.2% *vs* 22.1%).

The patients were categorized into three groups based on tumor size: Group 1 (tumor size ≤ 2 cm); group 2 (2-5 cm); and group 3 (≥ 5 cm). The total number of group 1 tumors was 62 [35 ER positive (56.5%); 3 PR positive (4.8%); and 3 HER2/neu positive (4.8%)]. The total number of group 2 tumors was 131 [55 ER positive (42.0%); 29 PR positive (22.1%); and 13 HER2/neu positive (9.9%)]. The total number of group 3 tumors was 66 [23 ER positive (34.8%); 10 PR positive (15.2%); and 7 HER2/neu positive (10.6%)]. ER expression in the HER2/neu positive, large-sized tumors was significantly decreased compared with smaller tumors ($P < 0.05$). Metastasis in axillary lymph nodes was seen in 237 (91.5%) patients. Among the ER positive cases, 86

Table 1 Clinico-pathological and molecular characteristics of the breast cancer patients (259)

Histologic type	Frequency	Percentage
Invasive ductal carcinoma	258	99.6
Invasive lobular carcinoma	1	0.4
Grading		
I	62	23.9
II	131	50.6
III	66	25.5
Tumour size		
< 2.0 cm	62	23.9
2-5 cm	131	50.6
> 5.0 cm	66	25.5
Lymph node involvement		
No	22	8.5
1-3	196	75.7
≥ 4	41	15.8
Hormonal receptors		
ER + ve	113	43.6
ER - ve	52	20.1
PR + ve	42	16.2
PR - ve	12	4.6
HER/2 neu + ve	23	8.9
HER/2 neu - ve	17	6.6
Total	259	100

ER: Estrogen receptor; PR: Progesterone receptor; Human epidermal growth factor receptor-2.

(43.9%) had axillary lymph node involvement ranging from 1-3. Among the PR positive cases, 37 (17.3%) had positive axillary lymph nodes positive for metastasis. Nineteen (9.7%) participants with HER2/neu positive disease were positive for axillary lymph nodes metastasis. Tumors with axillary lymph node metastasis showed significant association with ER, PR and HER2/neu ($P < 0.03$) (Table 2).

Age was significantly correlated with ER, PR and HER2/neu ($P < 0.05$). However, there was no significant correlation in PR negative disease ($P > 0.05$). We found that ER and HER2/neu expression compared to tumor grade was statistically significant ($P < 0.05$) while PR expression was not significant ($P > 0.05$) (Table 3).

This study also found that women younger than 50 years are likely to present with ER positive disease ($P = 0.01$, OR = 8.517, 95% confidence interval: 2.309-31.413) and PR negative disease ($P = 0.04$, OR = 14.00, 95% confidence interval: 2.300-85.218). Similarly, most of the tumors that presented with grade I disease were 15 times more likely to be ER positive than those in grade II or III ($P = 0.01$, OR 15.2, 95% confidence interval 1.296-12.252) (Table 4 and 5).

DISCUSSION

This study revealed that most of the patients (56%) were less than 50 years at diagnosis, and the mean age of the study participants was 48 years. This corresponds to what was reported by Sengal *et al*[17] and Errahhali *et al*[18] where the mean ages of the participants were 48 years. On the contrary, a similar study by Chand *et al*[19] reported a mean age of 55 years. In this study, the most predominant histologic type was invasive ductal carcinoma. A similar study in Sudan also reported invasive ductal carcinoma as

Table 2 Association of estrogen receptor and progesterone receptor with prognostic markers (*n* = 259)

Age	Hormonal receptors						Total	*P* value
	ER + ve	ER - ve	PR + ve	PR - ve	HER/2 neu + ve	HER/2 neu - ve		
< 50	73 (50.70%)	17 (11.80%)	34 (23.60%)	9 (6.30%)	8 (5.60%)	3 (2.10%)	144	0.01
≥ 50	40 (34.80%)	35 (30.40%)	8 (7.00%)	3 (2.60%)	15 (13.00%)	14 (12.20%)	115	
Grading								
I	35 (56.50%)	18 (29.00%)	3 (4.80%)	2 (3.20%)	3 (4.80%)	1 (1.60%)	62	0.02
II	55 (42.00%)	23 (17.60%)	29 (22.10%)	5 (3.80%)	13 (9.90%)	6 (4.60%)	131	
III	23 (34.80%)	11 (16.70%)	10 (15.20%)	5 (7.60%)	7 (10.60%)	10 (15.20%)	66	
Tumour size								
< 2.0 cm	35 (56.50%)	18 (29.00%)	3 (4.80%)	2 (3.20%)	3 (4.80%)	1 (1.60%)	62	0.02
2-5 cm	55 (42.00%)	23 (17.60%)	29 (22.10%)	5 (3.80%)	13 (9.90%)	6 (4.60%)	131	
> 5.0 cm	23 (34.80%)	11 (16.70%)	10 (15.20%)	5 (7.60%)	7 (10.60%)	10 (15.20%)	66	
Lymph node involvement								
No	11 (50.00%)	3 (13.60%)	3 (13.60%)	2 (9.10%)	2 (9.10%)	1 (4.50%)	22	0.03
1-3	86 (43.90%)	43 (21.90%)	34 (17.30%)	6 (3.10%)	19 (9.70%)	8 (4.10%)	196	
≥ 4	16 (39.00%)	6 (14.60%)	5 (12.20%)	4 (9.80%)	2 (4.90%)	8 (19.50%)	41	

ER: Estrogen receptor; PR: Progesterone receptor; HER/2 neu: Human epidermal growth factor receptor-2.

Table 3 Bi-variate correlation of clinicopathological parameters and hormonal receptors

Clinic pathologic feature	Estimators	Hormonal receptors					
		ER + ve	ER - ve	PR + ve	PR - ve	Her-2 + ve	Her-2 - ve
Age	R	-0.159[a]	0.231[b]	-0.224[b]	-0.086	0.131[a]	0.202[b]
	P value	0.01	0.01	0.01	0.167	0.036	0.001
Grading	R	-0.144[b]	-0.101	0.089	0.07	0.067	0.184[b]
	P value	0.015	0.087	0.133	0.236	0.26	0.002
Tumour size	R	-0.144[a]	-0.101	0.089	0.07	0.067	0.184[b]
	P value	0.015	0.087	0.133	0.236	0.26	0.002
Lymph node	R	-0.051	-0.018	-0.024	0.045	-0.047	0.184[b]
	P value	0.401	0.761	0.689	0.455	0.438	0.002
Total		259	259	259	259	259	259

[a]: Strongly significant.
[b]: Significant.
ER + ve: = Estrogen receptor positive; ER - ve = Estrogen receptor negative; PR + ve =Progesterone receptor positive; PR - ve =Progesterone receptor negative, HER/2 neu + ve= Human epidermal growth factor receptor-2 positive, HER/2 neu - ve= Human epidermal growth factor receptor-2 negative.

the most common histological type[17]. The majority of the ER (50%) and PR (23.6%) positive cases were seen in an age less than 50 years. This report agreed with other similar investigations in Sudan and the northern region of India[17,20]. HER2/neu positivity was observed in patients > 50 years. Our report was not in line with Alzaman *et al*[21] in Bahrain. There was a significant correlation between the age and ER, PR and HER2/neu positivity ($P < 0.05$). This was consistent with a similar study conducted by Ramic *et al*[22].

The majority of ER positive cases (57.0%) were observed in grade I carcinomas. Most PR positive cases (22.0%) were seen in grade II, and most HER2/neu positive cases (10.6%) were seen in grade III. This finding did not correspond to Siadati *et al*[23] where

Table 4 Multivariate logistic regression analysis of hormonal receptor and age patients

Hormonal receptors		*P* value	OR	95% confidence interval	
				Lower bound	Upper bound
ER + ve	< 50 yr	0.001	8.517	2.309	31.413
	≤ 50 yr	1			
ER - ve	< 50 yr	0.243	2.267	0.573	8.965
	≤ 50 yr	1			
PR + ve	< 50 yr	0	19.833	4.58	85.883
	≤ 50 yr	1			
PR - ve	< 50 yr	0.004	14	2.3	85.218
	≤ 50 yr	1			
HER/2 neu + ve	< 50 yr	0.238	2.489	0.548	11.308
	≤ 50 yr	1			

ER: Estrogen receptor; PR: Progesterone receptor; OR: Odd ration; HER/2 neu: Human epidermal growth factor receptor-2.

Table 5 Multivariate logistic regression analysis of hormonal receptor and tumor grading

Hormonal receptors	Grading	*P* value	OR	95% confidence interval	
ER + ve	Grade I	0.012	15.217	1.823	127.02
	Grade II	0.016	3.986	1.296	12.252
	Grade III	1			
ER - ve	Grade I	0.012	16.364	1.835	145.95
	Grade II	0.049	3.485	1.007	12.057
	Grade III	1			
PR + ve	Grade I	0.375	3	0.265	33.974
	Grade II	0.013	4.833	1.397	16.725
	Grade III	1			
PR - ve	Grade I	0.301	4	0.288	55.471
	Grade II	0.532	1.667	0.336	8.258
	Grade III	1			
HER/2 neu + ve	Grade I	0.246	4.286	0.366	50.197
	Grade II	0.105	3.095	0.789	12.144
	Grade III	1			

ER: Estrogen receptor; PR: Progesterone receptor; OR: Odd ration; HER/2 neu: Human epidermal growth factor receptor-2.

most of the ER positive cases were grade II[23]. A significant association was established between tumor grade, ER positivity and HER2/neu negativity ($P < 0.05$). A study conducted by Dodiya *et al*[24] in the western region of India showed that ER and PR correlated with the grade but not HER2/neu.

One hundred and thirty-one cases had a tumor size ranging from 2-5 cm. We observed that 42.0% were ER positive, 22.1% were PR positive and 9.9% were

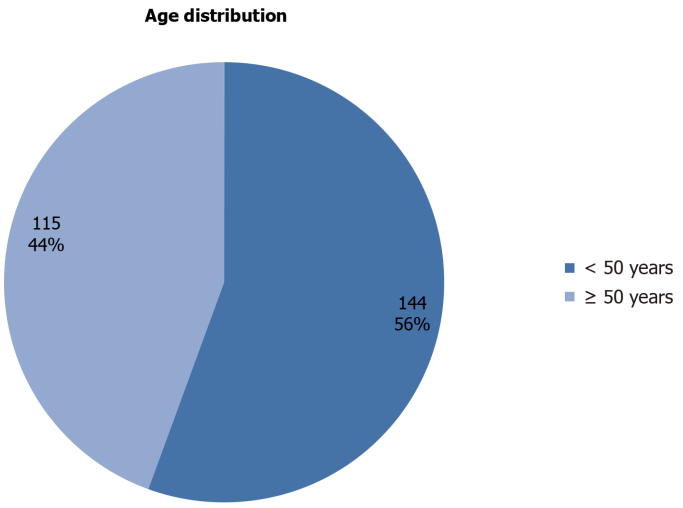

Figure 1 Age distribution of the study participants.

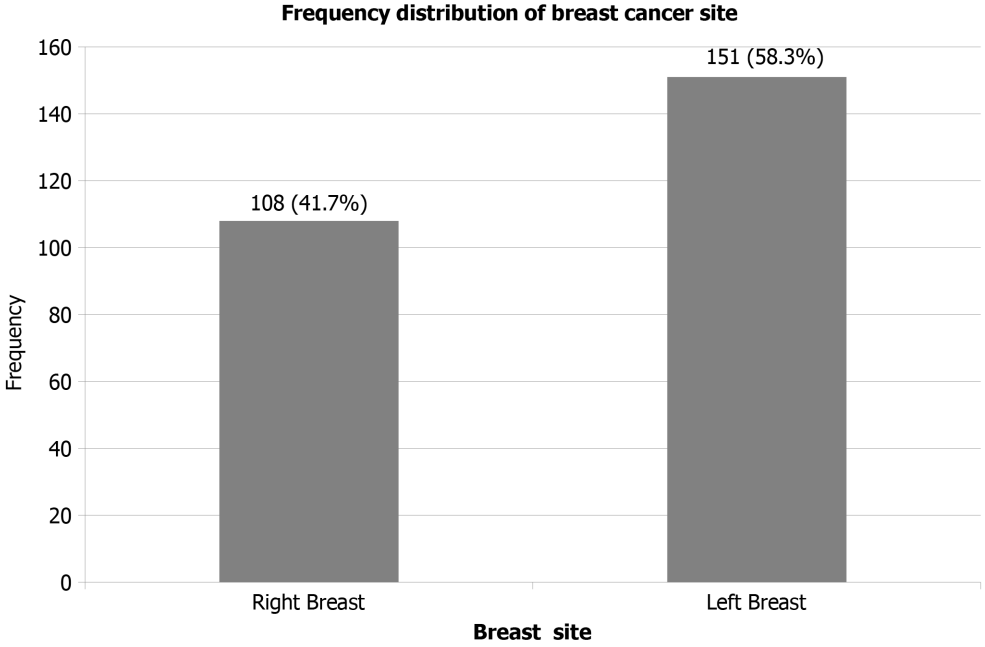

Figure 2 Frequency distribution of breast cancer site among the study participants.

HER2/neu positive. Our finding indicated that there was a negative correlation in ER positivity status and a positive correlation in HER2/neu positivity status with the tumor size (r = -0.144, P = 0.02). This agreed with a study by Almasri *et al*[25] that reported a significant correlation between tumor size and hormone receptors.

Metastasis in axillary lymph nodes was observed to be higher in ER positive cases (49.3%), while metastasis to axillary lymph nodes was present in 17.3% of the PR positive cases and 9.7% of HER2/neu positive cases. Tumors with axillary lymph node metastasis showed significant association with ER, PR and HER2/neu (P < 0.03). These findings agreed with other similar studies that showed a significant association between axillary lymph node and hormone receptors[19,26,27].

This study also found that women younger than 50 years were more likely to be present with ER positive and PR negative breast cancer. Similarly, most of the tumors that presented with grade I disease were 15 times more likely to be ER positive than those in grade II or III. On the contrary, Sengal *et al*[17] reported that younger women are 2 times more likely to develop ER negative disease, and grade III tumors are 2 times more likely to be ER negative compared with grade I or II tumors.

CONCLUSION

Our study showed that ER, PR and HER2/neu expression had a strong correlation with age, tumor grade, tumor size and lymph node status. Hence, hormone receptor assessment is highly recommended because of its significance in clinical management and prognostication.

ARTICLE HIGHLIGHTS

Research background

Female breast cancer is the leading type of cancer worldwide with an incidence of approximately 2.1 million in 2018. Hormone receptor status plays a vital role in its management.

Research motivation

Expression of biomarkers to treat breast cancer play a significant role in its management.

Research objectives

To determine the molecular expression pattern of biomarkers in breast cancer and their correlation with tumor variables.

Research methods

This prospective study was designed to analyze expression patterns of estrogen receptor(ER), progesterone receptor (PR) and HER2/neu in breast cancer patients. The dataset was taken from the Department of Radiotherapy and Oncology of Usmanu Danfodiyo University Teaching Hospital Sokoto, Nigeria from 1 January 2015 to 2 December 2019. The dataset had 259 records and 7 attributes. SPSS version 23.0 was used for statistical analysis. The data analyzed demographic and other clinicopathological characteristics as categorical variables. The mean and standard deviation were determined for the quantitative variable.

Research results

A total of 259 breast cancer cases were included in the study. The mean age was 48.3 ± 11.0, with an age range of 26-80 years and a median age of 46 years. The morphological categories were invasive ductal carcinoma (99.6%) and invasive lobular carcinoma (0.4%). Estrogen receptor positivity increased in 73 patients (50.0%) under the age of 50 years. Progesterone receptor positivity increase in 34 patients (23.6%) under the age of 50 years. Human epidermal receptor growth factor 2 positivity decreased in 8 patients (5.6%) under the age of 50 years. Hormone receptors were statistically significant with clinicopathological characteristics ($P < 0.05$).

Research conclusions

Our study showed that ER, PR and HER2/neu expression had a strong correlation with age, tumor grade, tumor size and lymph node status. Therefore, assessment of hormone receptors for clinical management of a breast cancer patient is strongly recommended to provide prognostic information and therapeutic measurement.

Research perspectives

In low-resource countries, there are paucities in the evaluation of the molecular expression of biomarkers in breast cancer. Therefore, there is a need for further studies to improve prevention and early detection of breast carcinoma in developing countries.

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ORIGINAL ARTICLE

Prospective Study

Functional Gait Assessment scale in the rehabilitation of patients after vestibular tumor surgery in an acute hospital

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Author contributions: Kos N drafted the manuscript, assisted with data analysis, and performed the statistical analysis; Brcar M participated in the design of the study, was involved in data collection, and assisted with data analysis; Velnar T participated in design and oversight of the study; all authors read and approved the final manuscript.

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Abstract

BACKGROUND

Patients in the acute phase of rehabilitation after vestibular tumor surgery are dysfunctional in basic daily activities. Balance, gait impairments, and falls are prevalent with vestibular loss.

AIM

To determine the degree of balance disorders after vestibular tumor surgery, the susceptibility to falls and to assess motor tasks using the Functional Gait Assessment (FGA) scale for functional gait, as part of the vestibular rehabilitation program during hospital stay.

METHODS

Patients who achieved a score higher than 25 points on the Mini-Mental State Examination and higher than 8 points on the Barthel index were included in the study. They were evaluated with the Berg Balance Scale the second day after surgery, during their hospital stay, at discharge, and three months after surgery. Throughout their hospitalization, patients took part in the vestibular rehabilitation program, focusing on multiple motor tasks included in the FGA.

RESULTS

All patients progressed clinically and statistically significant differences in functional activities of daily living were observed during hospitalization, before discharge to the home environment (median = 11; P = 0.0059) and three months after vestibular tumor surgery (median = 8; P = 0.0058). After discharge from hospital, four patients were at risk of falls, and two patients were at risk at three months.

Data sharing statement: There are no additional data available.

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CONCLUSION

Our study showed a positive effect of the use of FGA tasks as part of a rehabilitation program on functional activities of daily living in patients after vestibular tumor surgery. Nevertheless, we suggest further research to include a larger sample and a control group to overcome the deficiencies of our study.

Key Words: Vestibular tumor; Surgery; Assessment; Balance; Gait

Core Tip: This was a prospective pilot study of 10 patients who had problems with balance after surgical removal of a tumor from the cerebellopontine angle of the brain with the aim of using multitasking Functional Gait Assessment exercises as part of vestibular rehabilitation strategies for targeting better recovery and improvement of balance.

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INTRODUCTION

Vestibular schwannomas or acoustic neuromas are benign intracranial tumors of the vestibular nerve, which are usually treated by surgical resection[1,2]. The most commonly used surgical procedure in our clinical department is the retrosigmoid suboccipital approach[3]. It offers direct access to the tumor between the cerebellar hemisphere and the petrous bone with good visualization of the lower nerves and the inner auditory meatus, which is normally obscured by the tumor. The danger, however, is damage to the 7th and 8th nerve complex, which can be avoided by a meticulous operative technique and intraoperative monitoring[4,5].

In all cases, it is not possible to preserve the acoustic nerve, which is embedded in the tumor and is therefore destroyed during surgery. The facial nerve may be morphologically preserved[6,7]. Following resection, patients may experience vertigo, nausea, and a range of symptoms that include deficits in gaze stability, mobility, and balance[8,9]. Vestibular rehabilitation may be useful in reducing the severity and minimizing the impact of these symptoms[10,11]. Other problems, secondary to vestibular disorders, can also arise, such as nausea and vomiting, fatigue, and reduced ability to focus or concentrate[12]. Symptoms due to vestibular disorders can diminish the quality of life and impact all aspects of daily living. They also contribute to emotional problems such as anxiety and depression. Additionally, one of the consequences of having a vestibular disorder is that symptoms frequently cause people to adopt a sedentary lifestyle to avoid bringing on, or worsening, dizziness, and imbalance. As a result, decreased muscle strength and flexibility, increased joint stiffness and reduced stamina can occur[13,14].

Vestibular rehabilitation therapy (VRT) is a specialized exercise-based program primarily designed to reduce vertigo and dizziness, gaze instability, imbalance, and falls. For most patients with vestibular loss, the deficit is permanent as the amount of restoration of vestibular function is very small. However, after vestibular system damage, patients can feel better and function can return through compensation. This occurs because the brain learns to use other senses (vision and somatosensory; body sense) to substitute for the deficient vestibular system. The health of particular parts of the nervous system (brainstem and cerebellum, visual and somatosensory sensations) is important in determining the extent of recovery that can be gained through compensation. For many, compensation occurs naturally over time, but for patients in the acute stage of recovery, whose symptoms do not reduce and who continue to have difficulty returning to daily activities, VRT can help with recovery by promoting compensation[15].

The VRT goal is to use a problem-oriented approach to promote compensation. This is achieved by customizing exercises to address each patient's specific problems. Therefore, before an exercise program can be designed, a comprehensive clinical examination is needed to identify problems related to the vestibular disorder[16,17].

Depending on the vestibular-related problems identified, three principal methods of exercise can be implemented: (1) Habituation; (2) Gaze stabilization; and (3) Balance training[18].

Habituation exercises are used to treat symptoms of dizziness that are produced by self-motion or produced due to visual stimuli[19,20]. Habituation exercise is indicated for patients, who report increased dizziness when they move, especially when they make quick head movements, or when they change positions such as when they bend over or look up to reach above their heads. The goal of habituation exercises is to reduce the dizziness through repeated exposure to specific movements or visual stimuli that provoke patients' dizziness. These exercises are designed to mildly or at the most moderately, provoke the patients' symptoms of dizziness. The increase in symptoms should only be temporary, and before continuing onto other exercises or tasks the symptoms should return completely to the baseline level. Over time and with good compliance and perseverance, the intensity of the patient's dizziness will decrease as the brain learns to ignore the abnormal signals it is receiving from the inner ear[21].

Gaze stabilization exercises are used to improve control of eye movements so that vision is clear during head movement. These exercises are appropriate for patients who report problems seeing clearly because their visual world appears to bounce or jump around, such as when reading or when trying to identify objects in the environment, especially when moving around. There are two types of eye and head exercises used to promote gaze stability. The choice of which exercises to use depends on the type of vestibular disorder and extent of the disorder. One type of gaze stability exercise incorporates fixating on an object while patients repeatedly move their heads back and forth or up and down for a couple of minutes. The other type of gaze stability exercise is designed to use vision and somatosensation (body sense) as substitutes for the damaged vestibular system. Gaze shifting and remembered target exercises use sensory substitution to promote gaze stability. These exercises are particularly helpful for a patient with poor to no vestibular function[22].

Balance training exercises are used to improve steadiness so that daily activities for self-care, work, and leisure can be performed successfully. Exercises used to improve balance should be designed to address each patient's specific underlying balance problems[23]. Also, the exercises need to be moderately challenging but safe enough, to ensure that patients do not fall while doing them. Features of the balance exercises that are manipulated to make them challenging are also included in the Functional Gait Assessment (FGA) scale, which is focused on the movement of the head and trunk with vertical and horizontal turns during walking with specific activities requiring high-level function, such as gait on a level surface, change in gait speed, backward gait, step up and downstairs, stepping over obstacles, turning around, gait with eyes closed and gait with a narrow base of support. Additionally, the importance of FGA is increasingly emphasized during the patient's hospital stay and especially when they are released to the home environment[24,25]. Balance exercises should be designed to reduce environmental barriers and fall risk. For example, the exercises should help improve patients' ability to walk outside on uneven ground or walk in the dark. Ultimately, balance training exercises are designed to help improve standing, bending, reaching, turning, walking, and if required, other more demanding activities like running, so that patients can safely and confidently return to their daily activities[26,27]. Our main goal in this study was to use the FGA scale as part of vestibular therapy to improve the dynamic performances by new, learned strategies, that lead to the best optimal functional recovery. Due to the patient's balance impairment, relatively short hospitalization, and the fact that they are left on their own, leave without the possibility of rehabilitation treatment, the need for such a vestibular rehabilitation treatment, with included specific FGA activities during walking, should be initiated in the hospital.

Patients are seen by a specialist physiatrist and by a licensed physical or occupational therapist with advanced post-graduate training. Vestibular rehabilitation training begins the day after the patient's vestibular surgery with a comprehensive clinical assessment that includes collecting and documenting the type and intensity of postoperative symptoms and the precipitating circumstances. Acute stage rehabilitation aims to evaluate cognitive function[28], sensory and somatosensory abilities[29,30] and to prevent secondary complications[31], recover sensorimotor function, achieve interaction between postural control and selective movement to establish coordinated movement patterns and finally to achieve independence in the activities

of daily living and independence in performing functional gait[32,33]. In the acute post-surgery phase, gait function assessment is meant for clinical use only and is a part of functional assessment. For this purpose, the Barthel index is often used. It does not, however, provide adequate information/data for planning the rehabilitation program and measuring the functional outcome of the program[34]. Balance assessment is a part of the rehabilitation program in the early phase of rehabilitation and is implemented in different positions and activities, such as sitting, sitting to standing, and walking. The assessment of balance during gait is important for the identification of gait patterns and the introduction of suitable walking aids[35]. One of the most commonly used balance assessment tests in the hospital is a 14-item Berg Balance Scale (BBS), taken from the activities of daily living and represents the general mobility of the patients. The scale is organized in such a way that tasks move from less demanding to increasingly demanding functional abilities. The rating scale is for each of the five-level assignments (grades from 0 to 4). The total number of points is 56. The predictive value of the BBS for falls and the estimation of equilibrium indicate that scores below 45 points suggest an imbalance and thus an increased risk for falls. A clinically significant change between two BBS measurements indicating an improvement in equilibrium is 8 points[36,37].

As vestibular rehabilitation should focus on adaptation, particularly for the restoration of the dynamic functions, our focus was to improve patients' gaze of stability, balance control, and gait with challenging FGA tasks that require constant adjustment of postural stability. To confirm that statement, a more detailed analysis of the randomized-controlled trials investigating vestibular rehabilitation following the resection of an acoustic neuroma has provided greater insight into the efficacy of various components of vestibular rehabilitation interventions[38,39]. So far, we have not implemented such a demanding vestibular rehabilitation program in the hospital, and we would like to believe that the application of this type of high-level balance intervention during gait in patients could be useful in reducing the severity and minimizing the impact of their balance symptoms. Usually, physiotherapists treat patient's balance disorders in the acute hospital stage according to their methods and concepts, without implemented complex functional tasks, during gait. In patient's implementation of functional training in the early postoperative period, we try to eliminate, reduce or prevent defects of systems, that are important to the balance; develop an active balance-specific movement, sensory and cognitive strategy; and practice functional tasks by changing the complexity of posture tasks during standing and walking[40]. The regular hospital rehabilitation program does not implement various postural tasks included in the FGA, such as head-eye movements with various body postures and activities, and maintaining balance with a support base reduced with various orientations of the head and trunk, while performing gait and exposing patients gradually to various sensory and motor environments[41,42].

The purpose of this pilot study was to determine patients' balance disorders, their susceptibility to falls, and to investigate the acceptability of the BBS by reporting its score distribution (mean/mode score, minimal and maximal score). We also want to determine the adequacy of the FGA scale and its challenging tasks in patients after vestibular surgery during hospitalization.

MATERIALS AND METHODS

Patients and therapeutic procedures

A prospective nonrandomized study involving 10 patients admitted to the Department of Neurosurgery in Ljubljana from January 2016 to June 2017 for planned vestibular schwannoma surgery was conducted.

All patients underwent surgery using the retrosigmoid suboccipital approach, which is used in our clinical department. Before the operation, the patients were placed in the contralateral park-bench position and draped and prepped. The monitoring electrodes for all three facial branches were placed. The surgical approach then included osteoplastic or osteoclastic trepanation with exposure of the dura and transverse and sigmoid sinuses, and the bone was drilled over the sigmoid sinus to expose the mastoid air cells, which were sealed with bone wax. After relieving the cerebrospinal fluid (CSF) pressure through the opening of the cisterna magna, the tumor was visualized and devascularisation and detachment followed. In addition to the classical operative technique, an ultrasonic aspirator was also used. Monitoring was continuously carried out during the operation, to identify the facial nerve. In all cases, it was not possible to preserve the acoustic nerve, which was embedded in the

tumor and destroyed. The facial nerve was morphologically preserved. No postoperative complications were observed such as CSF leak, wound healing complications, or bone infection[6,7]. In all patients, tumor removal was complete.

The following inclusion criteria were taken into account: (1) Patient's first surgery of the cerebellopontine angle; (2) Ability to follow instructions; (3) Ability to participate (achieving at least 25 out of 30 possible in the Mini-Mental State Examination [MMSE]); and (4) Ability to walk the distance of 6 m without excessive fatigue with the help of a physiotherapist or a suitable walking aid (achieving at least 8 out of 20 possible in Barthel Index [BI])[28,34]. The MMSE and BI were carried out on the second day after surgery. Patients with pre-existing hemiplegia, paraplegia or paraparesis; previous head injury or lower limb damage; those with cardiorespiratory or febrile conditions and blind patients were excluded from the study.

In the first postoperative days, patients experienced dizziness, headache, and nausea. Before vestibular therapy, nausea, vomiting, and vertigo were prevented or reduced by medication, such as antiemetics or anti-vertigo drugs. According to a previous report[43], vestibular therapy must proceed from the head to locomotion in a top-down strategy; thus, the patients first performed Cawthorne-Cooksey (CC) exercises, which involved eye and head movements in the lying, sitting and standing position like as shown in (Table 1). The CC exercises are a graduated set of simple exercises to reduce dizziness and restore the patient' balance. They can also help to reduce sensitivity to motion, relax the neck and shoulder muscles, train the eyes to move independently of the head, practice the head movements that cause dizziness, help in the development of vestibular compensation, improve general coordination and encourage natural unprompted movement. The exercises were originally developed to help compensate for stable vestibular loss in one ear, such as following acoustic neuroma surgery. The purpose of these exercises was to build up a tolerance mechanism and the more diligently and regularly they were carried out by the patients, the sooner their symptoms would disappear[44]. In the beginning, all ten patients performed CC exercises three times a day for 10 min, with their eyes open and slow head movements in a supine position in bed, and only when they felt safe in a sitting and standing position, they moved to the next level of performing more challenging tasks involving dynamic stability of the body and limb movement. They then progressed to the next level with quick eye and head movements with their eyes closed. Patients reported mild to moderate discomfort while performing the exercises, also because of their awareness to endure dizziness. On the first day after surgery, we assisted patients in the exercises, and in the following days, they performed the exercises in the form of written instructions independently.

On the second day after surgery, when patients were able to walk the distance of 6 m (with the assistance of a physiotherapist or a suitable walking aid), the initial assessment of the patients' static and dynamic balance in the sitting and standing position was carried out using the BBS. On the day of discharge from hospital and three months after surgery, patients were re-tested using the BBS.

Based on the initial assessments of balance in the sitting and standing positions, the level of patients' balance impairments and the risk of falls were determined. Patients were divided into two groups according to the initial BBS results and their level of equilibrium impairment. Patients in the first group were those who reached a BBS value ranging from 21 to 40 points and had a truncated but acceptable balance; thus, they needed help during basic daily activities. These patients were able to walk only with a walking aid and the help of the physiotherapist. Patients in the second group included those with 41 or more points on the BBS and were independent during a short gait distance. However, postural instability and dynamic balance disorders were detected in patients performing functional gait. As none of the patients had an initial BBS score of more than 45 points, all patients were at risk for falls. They also completed the BBS assessment in full, with all the required tasks. Based on the BBS initial scoring, patients were included in a specially designed exercise protocol, with an emphasized requirement for postural adjustments and dynamic balance, while performing functional gait. The exercise protocol included 10 gait-related FGA activities and was organized as a 50 m marked polygon in the hallway section of the neurosurgery department, twice a day for 20 min with 5 repetitions of each functional item during a two-week hospital recovery period. The first group of exercises emphasized rotary head movement and body axis rotation; the second group emphasized a resizing of the support surface and stabilization of the body; the third group of exercises required a reduced proprioceptive inflow. All patients were motivated and focused on performing more demanding postural activities during exercise. No one suffered a fall, although the group with the poorer balance used a walking aid and physiotherapist assistance during functional gait. Patients in the

Table 1 The Cawthorne-Cooksey exercise order shown as it was during the performance of eye, head, and body movements in the supine, sitting, and standing position in all ten patients[43,44]

In bed–supine position	Sitting position	Standing position
Eye movements, head immobile, at first slow, then quick		
Up and down (1)	Repeat as the previous section	Repeat as in the previous section
Side to side (2)	Repeat as the previous section	Repeat as in the previous section
Repeat (1) and (2), focusing on finger	Shrug shoulders and rotate	Repeat as in the previous section
Focusing on the finger, moving about 3 feet to 2 inches away and back	Bend forward and pick up objects from the ground	Change from a sit to stand position with eyes open, then shut
Head movements, at first slow then quick, later with eyes closed		
Bending forward and backward	Rotate head and shoulders slowly, then fast; first with eyes open, then closed	Throw the ball from hand to hand (above eye level)
Turn side to side	Rotate head, shoulders, and trunk with eyes open, then closed	Throw the ball from hand to hand under knees
		Change from sitting to standing and turn around in between

second group were able to perform functional gait tasks independently under the therapist's supervision. Table 2 shows the functional FGA activities, performed by the patients in the first and second groups. The order of function tasks shown is as it was during exercise[41,42].

On patients' discharge from hospital to the home environment, they received relevant and thorough instructions on how to perform balance exercises at home in both printed and multimedia DVD format. The video clip was recorded outdoors-by the sea to bring the daily implementation of balance activities closer to the patient. Balance exercises for the home environment were composed of three sets; in a sitting and standing position and during gait. Each balance set consisted of 10 progressively challenging postural activities with five repetitions and progressed from stable positions to position changes and finally to upright positions, with various maneuvers to challenge balance, with eyes closed, feet together, picking up an object, turning, alternate stepping, narrowed base of support and functional gait. In addition, they were asked to fill in a specially designed logbook of balance exercise and a logbook of falls to provide feedback on the execution of therapeutic exercises and the potential occurrence of falls. Three months after surgery, all participating patients were invited for re-testing using the BBS.

The research was designed as a pilot study and will serve as a base for further research with a larger number of patients with the same diagnosis and a group of control patients with balance disorders. The patients will be treated by physiotherapists according to their concepts and methods, without challenging tasks during functional gait and their BBS results recorded. The BBS score the day before the operation will also be added as a control for all patients in the acute stage. The Medical Ethics Committee of the Republic of Slovenia approved the research design.

Data analysis

Descriptive statistics were calculated for the variables considered. The non-parametric Wilcoxon test for paired samples was used to compare the mean value of the BBS score at admission and discharge and three months thereafter. The statistical significance was set at $P = 0.05$. We used the R studio package (R version 3.6.1.).

RESULTS

Ten patients who underwent schwannoma surgery were included in the study: Six females and four males aged between 18 and 57 years (average 39.5 years). The average hospital stay was 10.5 d (minimum of 7 d, maximum 14 d).

Five patients had left-sided impairment, four had right-sided impairment and one patient did not exhibit clear lateralization of symptoms. The visual and auditory

Table 2 The purpose of the functional Functional Gait Assessment gait activities on the somatosensory system in all ten patients. The exercise order shown is as it was during exercise gait training[41,42]

Type of exercises	Purpose of the exercises
Walking with a glass full of water	Double attention
Walking with a change of speed	Stabilization of the body with a reduced support surface
Turning around its axis	Vestibular-ocular stabilization
Walking by turning the head in a horizontal plane (left/right)	Vestibular-ocular stabilization during walking
Walking by tilting the head in a vertical plane (up/down)	Vestibular-ocular stabilization during walking
Walking over obstacles	Thesis transfer and depth assessment
Walking backward	Decreased proprioceptive inflow
Walking with eyes closed	Decreased proprioceptive inflow
Tandem walking or walking heel toes forward	Reduced support surface
Walking up and downstairs	Thesis transfer and depth assessment

systems were affected in all participants: Five of them had suffered left-sided facial paralysis and deafness in the left ear, four had suffered right-sided facial paralysis, and deafness in the right ear, whereas one patient had symptoms of double vision and partial deafness in the left ear. The gustatory, olfactory, tactile, and vestibular systems were partially affected in all patients. Vestibular impairment in these patients consisted of disturbances in detecting changes in direction and speed of the head movement as well as changes in body position; acceleration, inhibition. Tactile impairment was shown to be a changed sense of touch, pain, pressure, and temperature. All patients had difficulties with swallowing, asymmetry in facial mimic, and speech problems as speech was not fluent and incomprehensible. Two patients experienced impairment of the proprioceptive system, and had trouble identifying the position and movement of the body or its parts. Despite all recorded sensory deficits, none of the patients reported any problems during testing and all of them completed the tests. The only symptom reported was early fatigue.

During the first BBS assessment, on the second day after surgery, none of the ten patients were able to perform gait without an adequate walking aid and they all needed help. Five patients who scored an initial BBS assessment ranging from 41 to 44 points required therapeutic readiness or supervision during gait; two patients needed an adequate walking aid with therapeutic supervision and scored 31 to 40 points on the BBS and; three patients scored 21 to 30 points on the BBS and needed a walking aid and help from the therapist during gait.

None of the patients exceeded the 46-point fall limit, although everyone was at risk for falls. After patient discharge from hospital to the home environment, six patients were able to perform gait without a walking aid; two needed supervision by the therapist and a suitable walking aid and two required an adequate walking aid and assistance from the therapist. Four patients were at risk for falls, and six exceeded the fall limit. Three months after surgery, functional gait in eight patients was independent and they were not at risk for falls, two patients still needed supervision during gait and were at risk for falls. Table 3 shows the use of walking aids, assistance by the therapist, and supervision during patients' gait on the first BBS evaluation (the fourth day after surgery), the second BBS assessment (at discharge to the home environment), and the third BBS assessment (three months after surgery).

Figure 1 shows the balance assessment using the BBS in each of the ten patients, during the first assessment (on the fourth day after surgery), second assessment (at discharge from the hospital), and third assessment (three months after surgery). In all patients, the progression assessment was statistically significant, both at the time of rehabilitation (median = 11; $P = 0.0059$) and three months after discharge (median = 8; $P = 0.0058$). The median progression was not statistically different ($P = 0.2012$). Figure 2 shows a flow chart of the progress of BBS assessment during rehabilitation treatment. Figure 3 is a diagram of the progress of BBS assessment three months after discharge from the hospital.

Table 3 The use of walking aids in patients during hospitalization and three months after surgery

Walking aid	First assessment (on the second day of surgery)	Second assessment (at discharge from hospital)	Third assessment (three months after surgery)
Without walking aid	0	6	8
Supervision	5	0	2
Walking aid and supervision	2	2	0
Walking aid and therapist assistance	3	2	0

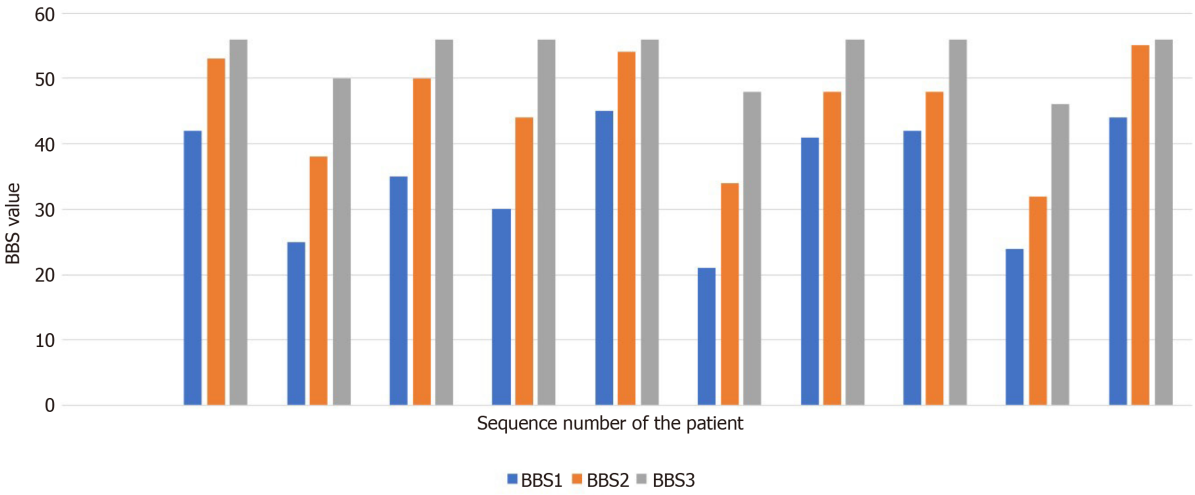

Figure 1 Berg Balance Scale balance assessment for each of the ten patients, during the first, second, and third Berg Balance Scale assessment. BBS: Berg Balance Scale.

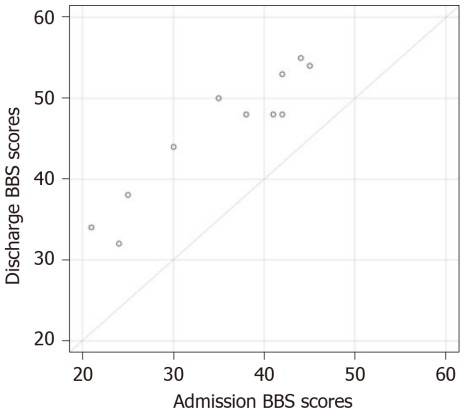

Figure 2 A diagram of patient progress in the Berg Balance Scale assessment during rehabilitation treatment. BBS: Berg Balance Scale.

DISCUSSION

In the early postoperative period, patients who have undergone vestibular tumor surgery experience balance impairments and postural instability, which are the main predictors of quality of life and have been shown to increase fall risk[45]. The most commonly used functional clinical assessment in patients with vestibular loss is the BBS. If the balance deficits in patients are identified early, the determination of appropriate vestibular treatment and effective strategies to prevent or decrease the negative effects of postural instability can be provided[46,47].

The purpose of our pilot study was to evaluate the improvement of functional balance in each patient and thus reduce the risk of falls in the hospital through the

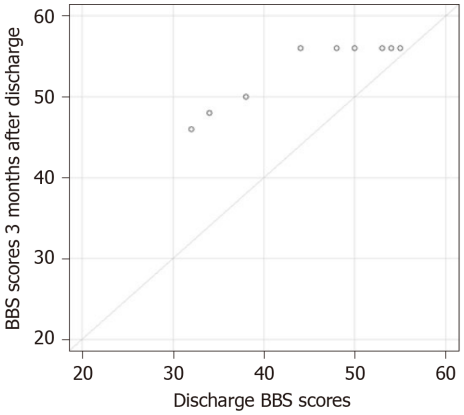

Figure 3 A diagram of the Berg Balance Scale assessment three months after discharge from hospital. BBS: Berg Balance Scale.

application of functional FGA exercises[48], which were implemented as part of the vestibular rehabilitation program to regain patients' ability to walk safely and to mimic their daily activities. Each movement challenging task emphasizes while performing functional gait, different aspects of the balance characteristics of patients with vestibular loss[49,50]. The vestibular rehabilitation interventions should be initiated in the early postoperative period of patients' recovery, as this critical plastic time window is characterized by major structural and functional changes. The efficacy of VRT might benefit from coinciding with the early stage of plastic event expression and may contribute to stabilizing, guiding, and shaping the newly formed functional connections in the deafferented vestibular nuclei and associated neuronal networks. This is in agreement with the top-down approach to vestibular compensation proposed by Balaban *et al*[51]. Adaptation, as a recovery mechanism of VRT, was used in our study and requires a dynamic interaction between the patient and the environment. This means that adaptation is a qualitative variation of the response, resulting from positive learning[52]. We can select a new reference frame for posture control and orientation perception and this can be done by re-weighting of the remaining sensory cues[53]. The lost dynamic vestibular function in patients can be replaced by new behavioral strategies involving several neuronal networks distributed in the brain, which reorganize to mimic the lost function[54]. Transferring newly learned skills with FGA functional gait training from the hospital into daily life is easier and faster if exercises are as close as possible to daily activities[55]. All patients in the study were motivated, fully focused, and were able to complete the FGA items. Ensuring patient safety during functional FGA activities was our major concern during treatment assessment[56,57].

The statistical analysis in our study showed a statistically significant functional balance improvement on the BBS scale at discharge from hospital (median = 11; P = 0.0059) and three months after discharge from hospital to the home environment (median = 8; P = 0.0058) in this sample of patients. This statistical analysis confirmed our hypothesis of the BBS scale being an appropriate functional balance assessment test in the early rehabilitation treatment, as it is also highly sensitive to changes that occur during rehabilitation programs and provides an adequate assessment of patients' postural instability.

In the initial BBS assessment, 30% of patients had very poor balance (BBS value from 21 to 30) and needed the help of a therapist and a walking aid during gait; 20% of patients had a truncated, but acceptable balance (BBS value from 31 to 40) and needed therapist supervision and an appropriate walking aid, and 50% of patients had good balance (BBS value from 41 to 45) and were independent during gait under the supervision of a physical therapist. All were at risk for falls. According to the initial BBS assessment, patients had difficulties in changing positions (such as positions with a smaller base of support or higher center of gravity) or in more challenging situations (such as increased range of motion or speed of movements). Patients with balance disorders require comprehensive assistance or more time to complete daily activities, thus they reduce the speed of performing daily tasks[45]. None of the patients were able to perform the most challenging items in the BBS, such as the "tandem stance", "one-legged stance", "alternating foot" and "look behind" items. The challenging items identified will help us better understand patients' balance decline sequence and guide screening and intervention programs[47].

The specificity of the chosen exercises was based on the principle of the autonomous phase of motor learning, the ability to focus on the influx from the proprioceptive system and the ability to implement motion strategies that, after a period of longer repetition, would almost completely become automatic, with a minimum degree of cognitive control. We predicted that patients would be able to transfer these newly learned movement strategies from exercise to daily life[55-57]. To achieve physical well-being we also advised them to take on some recreational activities such as dancing, yoga, or walking. Before discharge, the patients' family was acquainted with information on internal and environmental factors important for fall prevention. They were given instructions, both in written and multimedia format (DVD), regarding balance-improving exercises in the sitting and standing position and during gait. Additionally, they were asked to keep a record of home performing FGA exercises and all the falls in specially designed logs in their home environment. Before patients were discharged to the home environment, we detected a clinically significant change in improving balance in all 10 patients. According to the second BBS evaluation, 60% of patients scored 48 to 55 points and were independent during gait and not at risk for falls; 40% of patients scored 32 to 44 points on the BBS; they were at risk for falls and during gait needed a suitable walking aid and the therapist's assistance or supervision. Thus, 60% of patients evaluated using the BBS had high scores, which indicated the effect of the ceiling of the BBS scale. A ceiling effect occurs when the highest score on the scale does not capture or discriminate between differences in the upper and the attribute being measured[46,47]. Patients with the highest BBS scores were able to perform the most challenging BBS items such as tandem stance and one-legged stance.

Three months after discharge, according to the BBS estimate, 80% of patients had the highest score and a ceiling effect, which shows that the BBS functional test was not challenging enough to assess balance sufficiently and detect balance impairments across the full spectrum of patients with vestibular loss[57,58]. According to Steffen and Seney[59,60], the BBS score must change by at least 5 points to show a true change in balance. Therefore, 30% of patients after discharge and 70% of patients three months after discharge in our study were evaluated to have more than 52 points and were unable to show any progress in balance using the BBS. This lack of ability to identify balance impairments in patients with vestibular disorders three months after discharge may prevent important further intervention measures.

The BBS includes standing up, turning around and bending over, tandem stance, and one-legged stance, which are the most common ways falls occur in people after vestibular loss[46,47]. However, unlike the situations where falls occur, the BBS items allow full attention to be allocated to these tasks, possibly missing those who would lose their balance under nontested circumstances. It has been shown that attention allocation can drastically change balance deficits in patients after vestibular loss, whether it is a dual-task situation or the individual perceives that he or she is being observed[47]. The patients were fully focused and many were able to complete the BBS items, although these same tasks are problematic under normal circumstances.

This encouraged us to develop a special hospital rehabilitation program with FGA postural adjustments included while performing functional gait and can also have an improved impact on the more static items in the BBS.

Statistical analysis also showed that the median progression in balance after BBS did not differ significantly at the time of hospitalization and after discharge (P = 0.2012). This is the subject of further research with a control group of patients and, above all, a larger group of patients with vestibular disorders. We are not convinced of the significant impact on the progression of improvement of functional balance by the specifically designed FGA postural tasks in the home environment and the impact of normal recovery in patients after vestibular surgery.

CONCLUSION

Implemented multitasking FGA exercises, as a part of the vestibular rehabilitation program for functional gait, proved helpful in the acute period of hospital stay as the functional independence of patients while engaging in basic daily activities improved at the time of discharge and three months after discharge. The BBS is considered the reference standard for the determination of fall risk and evaluation of balance impairments in patients after vestibular tumor surgery. The FGA dynamic gait items were used as a part of the vestibular rehabilitation program to improve the patients' static BBS items and to determine their internal and critical external risk factors for falls. However, given the small number of cases and lack of a control group, we cannot

with certainty conclude that the FGA Scale tasks provide sufficient positive effects on early-stage rehabilitation in patients after vestibular tumor surgery. We believe future research with a larger sample size and the inclusion of a control group would provide an insight into the effects of the implementation of the FGA scale tasks on early-stage rehabilitation programs and might also provide useful information on the guidelines for early stage rehabilitation in patients after vestibular tumor surgery.

ARTICLE HIGHLIGHTS

Research background

Patients in the acute phase of rehabilitation after vestibular tumor surgery usually experience vertigo, nausea, and a range of symptoms that include deficits in gaze stability, mobility, and balance. In addition to these problems, secondary problems such as vomiting, fatigue and reduced ability to focus or concentrate can occur. These symptoms can diminish the quality of life and impact all aspects of daily living and contribute to emotional problems sometimes causing anxiety or depression. Due to these symptoms, relatively short hospitalization, and the fact that they are left on their own, without the possibility of rehabilitation treatment, there is a need for such a vestibular rehabilitation treatment, including specific Functional Gait Assessment (FGA) activities during walking, and should be initiated in the hospital.

Research motivation

The purpose of our pilot study was to evaluate the improvement in functional balance in each patient and thus reduce the risk of falls. Experimental functional FGA exercises were chosen as a part of the vestibular rehabilitation program to regain patients' ability to walk safely and to mimic their daily activities. Furthermore, this topic was chosen as the regular hospital rehabilitation program does not implement various postural tasks included in the FGA. We believe future research should focus on attempting to develop the most effective vestibular rehabilitation program for patients with vestibular loss in the early postoperative period.

Research objectives

The main goals of this study were to improve dynamic performance, to determine patients' balance disorders; their susceptibility to falls, and to investigate the acceptability of the Berg Balance Scale (BBS) by reporting its score distribution. Most of the patients in the study, evaluated with the BBS showed statistically significant clinical progress in functional activities of daily living. The routine collection and reporting of BBS balance outcomes before and after surgery should be considered for all patients with vestibular loss, given the profound impact on the overall quality of life and for further quality studies in this area.

Research methods

Our study was conducted at the Department of Neurosurgery in Ljubljana between January 2016 and June 2017. We included patients who underwent vestibular schwannoma surgery and scored higher than 25 points on the Mini-Mental State Examination and higher than 8 points on the Barthel index during the initial examination. We used the BBS for evaluation on the second day after surgery, during their hospital stay, at discharge, and three months after surgery. A vestibular rehabilitation program, focusing on multiple motor tasks included in the FGA scale was formed and implemented in the acute stage of rehabilitation. Before discharge, the patients were provided with instructions regarding balance-improving exercises in sitting and standing positions and during gait. Additionally, they were asked to keep a record of FGA-based exercise performance as well as falls in their home environment. Descriptive statistics were used for the variables considered. The non-parametric Wilcoxon test for paired samples was used to compare the mean value of the BBS scale at admission and discharge and three months after discharge. The statistical significance was set at $P = 0.05$. We used the R studio package (R version 3.6.1.).

Research results

Ten patients were included in the research: Six females and four males aged between 18 and 57 years (average 39.5 years). The average hospital stay was 10.5 d (minimum of 7 d, maximum 14 d). Analysis showed a statistically significant improvement in functional balance on the BBS scale at discharge from hospital (median = 11; P =

0.0059) and three months after discharge from hospital (median = 8; P = 0.0058) in these patients. The statistical analysis confirmed our hypothesis that the BBS scale is an appropriate functional balance assessment test in early rehabilitation, as it is also highly sensitive to changes that occur during rehabilitation programs and provides an adequate assessment of patients' postural instability. Statistical analysis also showed that the median progression in balance after BBS did not significantly differ at the time of hospitalization and after discharge (P = 0.2012). Larger numbers and more homogeneous cases in potential future studies would confirm or refute the results of our study.

Research conclusions

Multitasking FGA exercises, as a part of the vestibular rehabilitation program for functional gait, proved helpful in the acute postoperative period as the functional independence of patients improved at the time of discharge and three months after discharge. However, given the small number of cases and lack of a control group, we cannot with certainty conclude that FGA scale tasks provide sufficient positive effects on early-stage rehabilitation in patients after vestibular tumor surgery. There are several key priorities for future research. Randomized controlled trials with larger sample sizes and more rigorous methodologies are needed to investigate the effects of each motor element of the FGA scale. Investigation of FGA intervention parameters, based on routine collection of balance outcomes, the timing and duration of FGA interventions, and the minimum or optimal "dosage" requirement to achieve effectiveness should be considered. The effects of education and social reinforcement also require further investigation.

Research perspectives

Our study may be a good baseline for further research and to establish the Slovenian rehabilitation register for patients after surgical removal of vestibular tumors. The register should include a valid rehabilitation protocol for all Slovenian hospitals in primary care.

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CASE REPORT

Chemotherapy rechallenge in metastatic colon cancer: A case report and literature review

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Abstract

BACKGROUND

Colorectal cancer (CRC) is the third leading cause of cancer-related death in males and females in the United States. Approximately, 20%-22% of patients have metastatic disease at the time of presentation, and 50%-60% will develop metastasis over the course of their disease. Despite advances in systemic therapies, there remains a paucity of effective third- and later-line therapies for patients with ongoing disease progression. However, rechallenging chemo-resistant CRC tumors with previously administered therapies is an emerging concept that may be a life-prolonging option for heavily treated metastatic colorectal cancer (mCRC).

CASE SUMMARY

A 41-year-old man with no previous medical history initially presented with worsening diffuse abdominal tenderness. Computed tomography was significant for a splenic flexure mass and hepatic lesions concerning for metastatic disease. He underwent a colectomy with anastomosis. Postoperative pathology was diagnostic for moderately to well-differentiated adenocarcinoma (T4bN1bM1a). He received adjuvant 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX), but therapy was discontinued due to the development of atrial fibrillation. Additional workup indicated a carcinoembryonic antigen level of 508.2 ng/mL, and mutational analysis found that the tumor was microsatellite instability-high and

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KRAS/*BRAF* wild-type. He was started on irinotecan with oxaliplatin (IROX), and bevacizumab (14 cycles), developed disease progression, was transitioned to FOLFOX and cetuximab, and then eventually three cycles of pembrolizumab. Following disease progression, he was rechallenged with IROX therapy, as he previously responded well to oxaliplatin-based therapy. The IROX rechallenge provided this patient with a ten-month survival benefit, decreased metastatic burden, and marked improvement in his clinical condition.

CONCLUSION
Rechallenge of previous lines of well-tolerated systemic chemotherapy regimens may be a valuable therapeutic strategy in patients with heavily-treated mCRC.

Key Words: Metastatic colorectal cancer; Rechallenge therapy; Treatment holiday; Oxaliplatin; Irinotecan; Case report; Chemoresistance; Palliative option

Core Tip: Despite advances in therapeutic strategies, colorectal cancer (CRC) remains a deadly disease. There are limited options for patients with chemo-refractory mCRC. We present a case of a patient with heavily treated metastatic colorectal cancer (mCRC) that was responsive to oxaliplatin-based rechallenge therapy. Oxaliplatin rechallenge therapy provided this patient with a ten-month survival benefit, marked improvement in his clinical condition, performance status, and quality of life. This case highlights the importance of considering rechallenge therapy in patients with chemo-refractory mCRC. Monitoring for oxaliplatin-associated peripheral sensory neuropathy should be considered for patients who are candidates for oxaliplatin rechallenge therapy.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death in the United States[1]. An estimated 147950 new CRC cases and 53200 deaths are expected to occur in 2020[1]. Approximately 20%-22% of CRC patients present with metastatic disease at initial diagnosis and 50%-60% will develop metastasis throughout their disease[1]. Metastatic colorectal cancer (mCRC) carries a poor prognosis, with a 5-year overall survival (OS) rate of 14%[1]. The current recommended first-line systemic chemotherapy regimens include 5-fluorouracil, leucovorin, irinotecan (FOLFIRI), 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX), capecitabine plus oxaliplatin (XELOX), and 5-fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI)[2-5]. Biologic agents such as the vascular endothelial growth factor inhibitor bevacizumab and epidermal growth factor receptor (EGFR) inhibitor cetuximab may be used as adjuncts to systemic chemotherapy in mCRC[6,7].

Despite advances in cytotoxic and targeted therapy, treatment resistance remains a significant barrier to the management of mCRC. Resistance can be primary (poor initial response) or secondary (loss of initial response). Many patients exhibit rapid disease progression with third- and fourth-lines therapies. The therapeutic options for these patients remain limited and typically consist of regorafenib or trifluridine-tipiracil (FTD/TPI)[8,9]. The role of rechallenge therapy with chemotherapy, biologic agents, or combination therapy in patients who have developed secondary resistance, particularly previously responding patients, is not clear. We present a case that challenges the dogma of irreversible secondary resistance and supports the potential of rechallenge chemotherapy as a life-prolonging treatment option in heavily-treated

mCRC.

CASE PRESENTATION

Chief complaints

A 41-year-old man presented for a second opinion regarding his worsening abdominal pain.

History of present illness

The patient initially presented to a hospital in Qatar with an 8-mo history of diffuse abdominal pain. A computed tomography (CT) scan and colonoscopy revealed a splenic flexure mass and diffuse hepatic lesions concerning for metastatic disease. The patient then received another opinion at a hospital in Bangkok, Thailand, where a repeat CT scan and colonoscopy confirmed the initial diagnosis. The patient had a 17 pack-year cigarette smoking history. He denies alcohol consumption or a family history of cancer. During his clinical visits in Qatar and Thailand, his physical exam was unremarkable, and he denied weight loss, constipation, diarrhea, hematochezia, or melena. In Qatar, he underwent a colectomy with anastomosis. The pathologic diagnosis was moderate to well-differentiated colonic adenocarcinoma, stage T4bN1bM1a. He then received adjuvant treatment with FOLFOX chemotherapy, however after the first cycle, he developed atrial fibrillation, and treatment was discontinued. Afterward, he presented to our hospital for further workup and recommendations.

History of past illness

The patient has no past medical history.

Personal and family history

The patient has no personal or family history.

Physical examination

The vitals on admission was within normal reference range and his physical examination was unremarkable.

Laboratory examinations

Labs indicated a normocytic anemia, and an elevated carcinoembryonic antigen (CEA) level (508.2 ng/mL). Blood chemistries, urinalysis, urine cultures, coagulation times including international normalized ratio, prothrombin, and partial thromboplastin times were within normal limits. Electrocardiogram was within normal limits.

Imaging examinations

An initial imaging evaluation with CT of the abdomen and pelvis revealed two hypoattenuating masses in the liver. One 6.1 cm superior mass was in the right lateral lobe of the liver and a 6.9 cm inferior mass was in the lower tip of the right lobe (Figure 1). These masses were most compatible with liver metastasis from invasive CRC. The portal vein was patient, bile ducts were not dilated, and the gallbladder was normal.

Further diagnostic workup, diagnosis, disease management, and treatment

Pathology slides of tumor tissue samples were obtained from the patient's hospitalization in Bangkok and reviewed. The pathology report indicated invasive colonic adenocarcinoma, moderately differentiated (low-grade). The mutational analysis found that the patient had high microsatellite instability and wildtype (WT) *KRAS* and *BRAF* mutations. Given his history of atrial fibrillation on FOLFOX, he was started on irinotecan and oxaliplatin (IROX) plus bevacizumab. The patient had a partial response to IROX plus bevacizumab therapy but after 14 cycles of treatment, he developed disease progression and was transitioned to FOLFOX plus cetuximab. His disease progressed on FOLFOX and cetuximab, and thereafter the patient was lost to follow up for one year. During that time, he received three cycles of pembrolizumab from an outside hospital. Unfortunately, staging workup after 3 cycles of pembrolizumab indicated enhanced disease progression, particularly increased enlargement of metastatic liver lesions. In early 2018, we discussed with the patient about three potential chemotherapy options: (1) FTD/TPI; (2) Regorafenib; and (3)

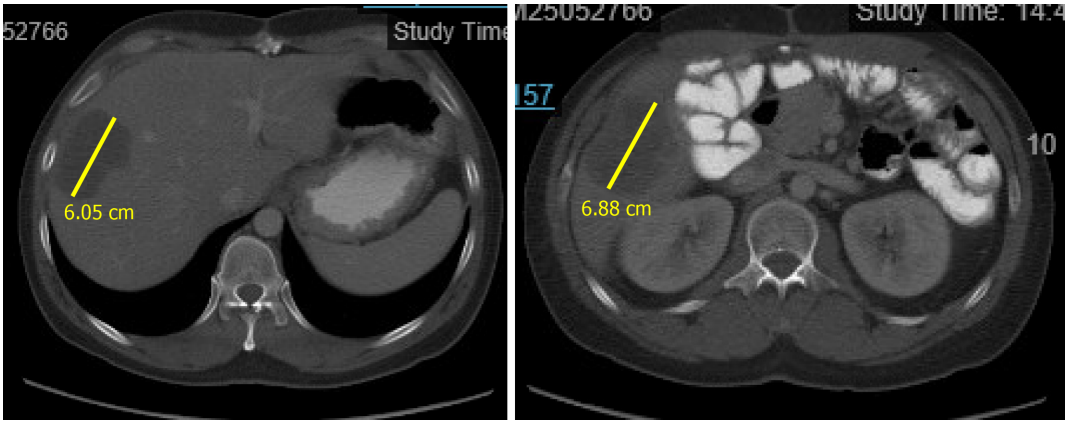

Figure 1 Initial computed tomography images of abdomen and pelvis displaying two hypoattenuating liver masses, which are most consistent with liver metastases from invasive colorectal carcinoma. Images were captured on 7/25/2016.

Rechallenge with IROX. The decision was made to rechallenge with IROX therapy based on the patient's previous response to this regimen. Therefore, the patient received rechallenge therapy with IROX plus cetuximab every two weeks (11 cycles total). He tolerated the treatment well and showed a marked improvement in his clinical condition, performance status, and quality of life. Furthermore, a substantial reduction in the CEA level from 4493 ng/mL to 2250 ng/mL was observed after four months of treatment (Figure 2). Follow-up CT scans showed a decrease in liver metastasis size shown in the scans obtained at five and six months after rechallenge therapy (Figure 3). The diagnostic workup, disease management, and treatment are also summarized in Figure 4.

FINAL DIAGNOSIS

The final diagnosis was chemo-resistant metastatic *KRAS/BRAF* WT, microsatellite instability-high colorectal carcinoma (T4bN1bM1a).

TREATMENT

The patient continued to receive IROX plus cetuximab rechallenge chemotherapy for a total of nine months. The treatment was eventually withheld due to hospital admission for pneumonia and respiratory failure.

OUTCOME AND FOLLOW-UP

After noting treatment complications, the patient and his family chose to transition to palliative care and hospice. He passed away 27 mo after his initial presentation to our hospital. The oxaliplatin rechallenge therapy provided this patient with a ten-month survival benefit.

DISCUSSION

Limited cytotoxic agents are available for the treatment of mCRC. Unfortunately, there are a significant number of patients that still progress past the third and fourth line of therapy, who may respond to other therapeutic options. Rechallenge therapy with either chemotherapy/biologic therapy alone or combination therapy has not been fully investigated as a viable option for patients with disease progression. In this case, oxaliplatin-based chemotherapy rechallenge demonstrated a viable alternative for this patient with heavily-pretreated mCRC. Despite progression on multiple lines of chemotherapy, immunotherapy, and investigational therapy, IROX rechallenge provided this patient with an additional ten-month survival benefit. The rationale of

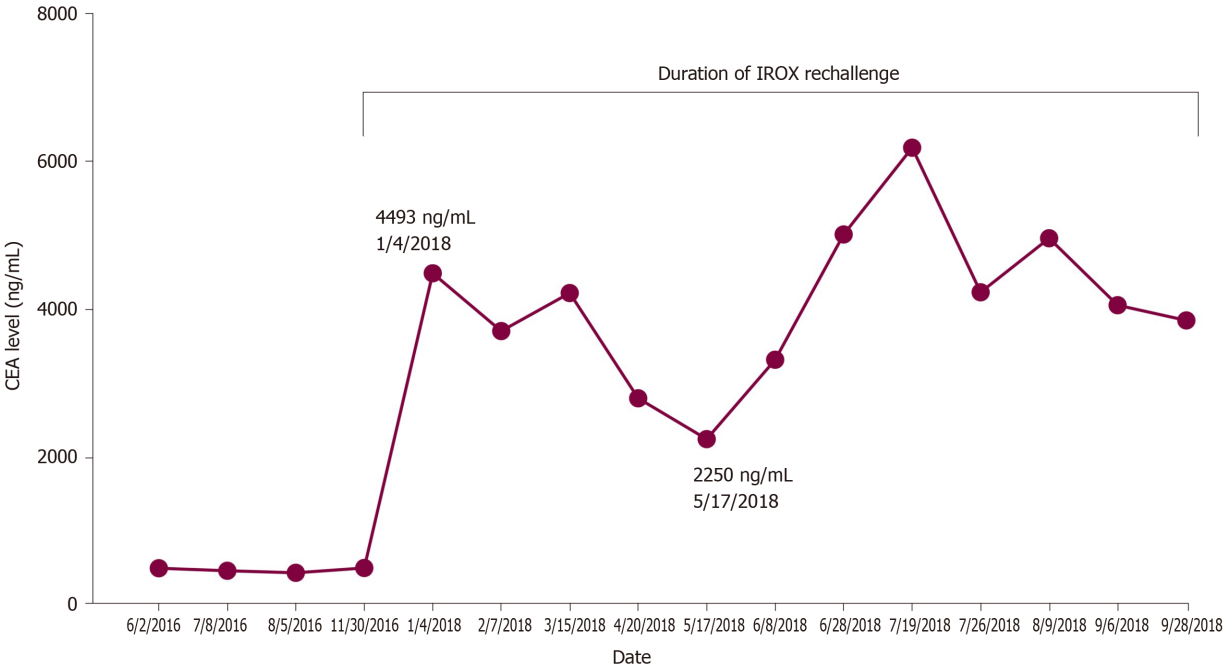

Figure 2 Trend in carcinoembryonic antigen levels during irinotecan with oxaliplatin rechallenge therapy. CEA: Carcinoembryonic antigen; IROX: Irinotecan with oxaliplatin.

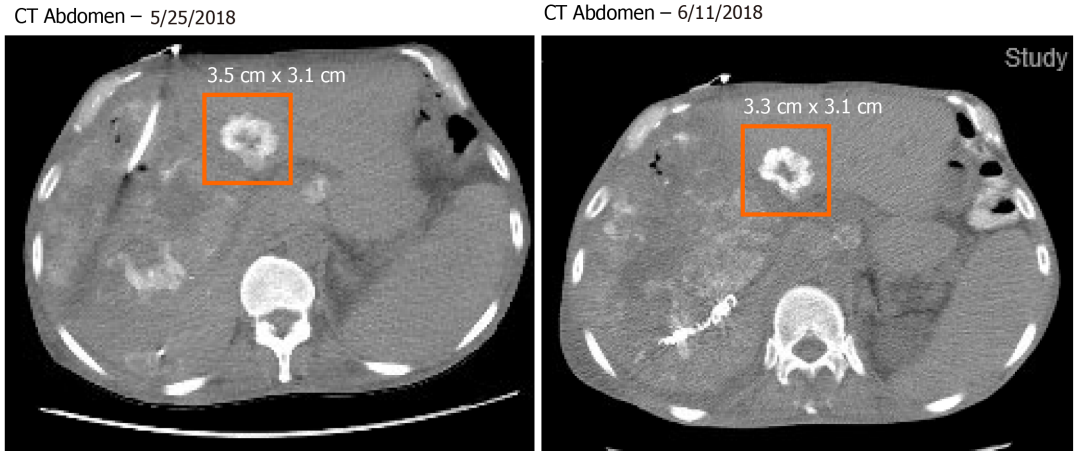

Figure 3 Computed tomography abdomen images showed interval response with decrease in liver metastasis size. These images show size of the metastatic lesion at 4- and 5-mo post initiation of irinotecan with oxaliplatin rechallenge therapy. CT: Computed tomography.

reattempting a previous line of systemic chemotherapy stems from the possibility that subsequent therapies following the development of chemoresistance may sensitize patients to the primary therapy by promoting the growth of sensitive clones[10]. Another possibility is epigenetic alterations may result in tumor resistance, which may reverse following a "drug holiday"[10]. While mechanisms that result in chemoresistance are well known, the cellular mechanisms and predictive factors associated with response to rechallenge therapy need to further elucidated.

Findings from this case are consistent with previous studies demonstrating the promise of oxaliplatin-based chemotherapy rechallenge in the third- or fourth-line setting for mCRC. These previous studies are summarized in Table 1. Suenaga *et al*[11] performed a single-arm, open-label, phase II clinical trial (RE-OPEN) to examine the safety and efficacy of reintroducing oxaliplatin in patients with mCRC refractory to standard chemotherapy. The eligible patients in this study had previously received oxaliplatin and irinotecan and achieved stable disease or response, followed by disease progression ≥ 6 mo during the first oxaliplatin-based therapy. The primary endpoint was disease control rate (DCR) after 12 wk of re-challenge therapy and the secondary endpoints were safety, overall response rate, and progression-free survival (PFS).

Table 1 Summary of studies assessing the efficacy of oxaliplatin-based rechallenge therapy against metastatic colorectal cancer

Ref.	Prior lines of chemotherapy	Patient cohort	Treatment regimen	PFS (months)	OS (months)
Suenaga *et al*[11]	Oxaliplatin-based therapy: First-line: 26 (78.8%); Second-line 7 (21.2%). Molecular-targeted therapy: None: 1 (3.0%); Bevacizumab: 27 (81.8%); Cetuximab or Panitumumab: 19 (57.6%)	33 patients, previously received oxaliplatin and irinotecan, had stable disease, and disease progression ≥ 6 mo	FOLFOX6: 33 (100%)	3.2	9.8
Yang *et al*[13]	Oxaliplatin-based therapy: First-line: 76 (80.0%); Second-line 19 (20.0%). Oxaliplatin rechallenge therapy: Third-line: 78 (82.1%); Fourth-line: 13 (13.7%); Fifth or more: 4 (4.2%). Control arm: Anti-EGFR + irinotecan: 29 (100%)	Rechallenge arm: 95 patients received who received oxaliplatin in the first/second-line and were rechallenged as a third or later line of therapy	mFOLFOX6: 70 (73.7%); XELOX: 19 (20%); Other: 6 (6.3)	1.7[a]	12.2
		Control arm: 29 patients received anti-EGFR and irinotecan therapy	Anti-EGFR + irinotecan: 29 (100%)		11.4
Köstek *et al*[14]	All patients received two lines of chemotherapy of any of the following combinations: (FOLFIRI, FOLRFIRI/XELIRI, FOLFOX/XELOX, capecitabine, FUFA/capecitabine) monotherapy or combined with biologic agents bevacizumab/cetuximab/panitumab	Regorafenib arm: 73	Regorafenib	3.4	6.6
		Rechallenge arm: 31; rechallenge therapy was identified as re-using a regimen that was previously administered to patients and had obtained disease control	FOLFOX + cetuximab: 8, FOLFOX + bevacizumab: 6, FOLFOX: 4, FOLFIRI: 2, FOLFIRI + cetuximab: 3, capecitabine: 2, FOLFOX + panitumab: 1, FOLFIRI + bevacizumab: 1, FUFA + bevacizumab: 1, capecitabine + bevacizumab: 1, XELOX + bevacizumab: 1, FOLFIRINOX: 1	9.2	12.0
Fernandes *et al*[16]	Patients with documented progression to regimens containing oxaliplatin, irinotecan, and 5-FU (most patients received at least three regimens)	Rechallenge arm: 21 patients who were rechallenged with either FOLFIRINOX or FOLFOXIRI	FOLFIRINOX: 13 (61.9%); FOLFOXIRI: 8 (38.1%)	4.0	8.6
Townsend *et al*[17]	Patients identified to received oxaliplatin previously. Prior lines of chemotherapy before oxaliplatin rechallenge (information on prior regimen was not provided): 4 lines for 2 patients, 3 lines for 6 patients, 2 lines for 8 patients, 1 line for 4 patients	Rechallenge arm: 20 patients who were rechallenged with FOX therapy	FOX: 20	3.7	7.8
Sgouros *et al*[18]	Patients previously treated with IROX with refractory disease (median prior lines of chemotherapy: 3)	Rechallenge arm: 25 patients who were rechallenged with IROX therapy	Irinotecan (180 mg/m^2/135 mg/m^2) and oxaliplatin (85 mg/m^2/65 mg/m^2) every two weeks	3.0	7.0

[a]Best PFS for all oxaliplatin based rechallenge regimens evaluated.

FOLFOX: Folinic acid, 5-fluorouracil, and Oxaliplatin; EGFR: Epidermal growth factor receptor; XELOX: Capecitabine and oxaliplatin; FOLFIRI: Folinic acid, 5-fluorouracil, and irinotecan; XELIRI: Capecitabine and irinotecan; FUFA: Folinic acid and 5-fluorouracil; FOX: Oxaliplatin and fluoropyrimidine; 5-FU: 5-Fluorouracil; IROX: Irinotecan with oxaliplatin; FOLFOXIRI: 5-Fluorouracil, leucovorin, oxaliplatin, and irinotecan; FOLFIRINOX: Oxaliplatin, irinotecan, fluorouracil, and leucovorin. PFS: Progression free survival; OS: Overall survival; NR: Not reported.

Oxaliplatin was reintroduced by treating patients with the FOLFOX6 regimen. The study found that the DCR after 12 wk of rechallenge therapy was 39.4% (95%CI 21.8-57.0) and the response rate (complete and partial response) was 6.1%. The median PFS and OS were 3.2 and 9.8 mo, respectively. A concern with rechallenging patients with oxaliplatin is the risk of developing peripheral sensory neuropathy (PSN) during the oxaliplatin rechallenge. However, in this study, the incidence of grade 1 and 3 PSN events was 53.1% and 0%, respectively[11].

A follow-up to the RE-OPEN trial was a phase I clinical trial (LUPIN study), in which the study examined the safety of rechallenging oxaliplatin with FTD/TPI[12]. The patient cohort of interest in this study were patients with mCRC, whose tumors acquired resistance to prior chemotherapy with oxaliplatin and irinotecan[12]. The study concluded that a safe rechallenge regimen would be oxaliplatin 85 mg/m^2 on days 1 and 15 every four weeks and FTD/TPI 35 mg/m^2 bid on days 1-5 and 15-19. The LUPIN study concluded that FTD/TPI could potentially replace 5-fluorouracil in combination with oxaliplatin. The efficacy of this novel combinatorial approach of FTD/TPI with oxaliplatin for mCRC needs to be further evaluated.

Comparable results were found in a retrospective study performed by Yang *et al*[13]

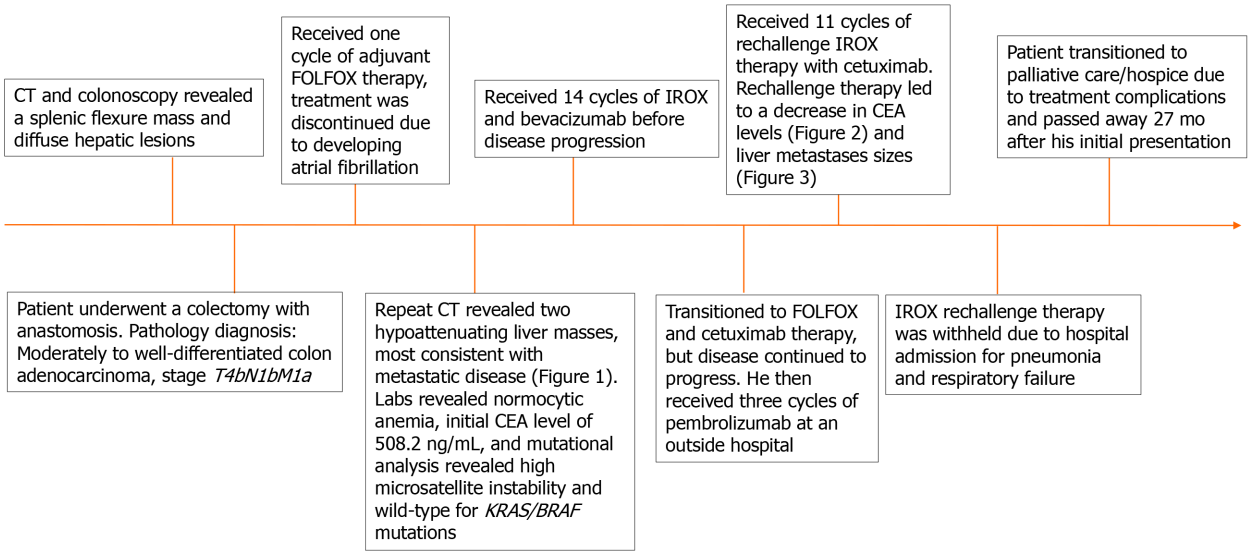

Figure 4 Timeline summary of events. CT: Computed tomography; FOLFOX: Folinic acid, 5-fluorouracil, and oxaliplatin; CEA: Carcinoembryonic antigen; IROX: Irinotecan and oxaliplatin; *KRAS*: Kirsten rat sarcoma viral oncogene; *BRAF*: B-Raf oncogene.

in 2018. In this study, patients with mCRC, who progressed from oxaliplatin, fluoropyrimidine, and irinotecan as first and second-line chemotherapy, were re-challenged with an oxaliplatin-based therapy. The control arm in this study was mCRC patients treated with anti-EGFR biologic therapy and irinotecan. The OS for oxaliplatin rechallenge arm and control arm was 12.2 and 11.4 mo, respectively (no significant difference between both treatment arms, $P > 0.05$). Multivariate analysis found that patients who obtained disease control with oxaliplatin rechallenge had a better time to treatment failure (6.1 *vs* 1.7 mo, $P < 0.001$) and OS (15.7 *vs* 6.3 mo, $P < 0.001$) compared to patients with progressive disease. This study showed that rechallenge with oxaliplatin-containing chemotherapy yielded equivalent tumor control and survival benefit to that of anti-EGFR antibodies with irinotecan in the third- or later-line setting in mCRC[13].

Köstek *et al*[14] found that chemotherapy rechallenge (FOLFOX alone, FOLFOX with either cetuximab or bevacizumab, FOLFIRI alone or with cetuximab or bevacizumab, capecitabine plus bevacizumab, FOLFIRINOX, XELOX plus bevacizumab, or folinic acid and 5-fluorouracil plus bevacizumab) was more effective than regorafenib in the third-line treatment of mCRC patients. The PFS and OS with rechallenge therapy were 9.2 mo and 12 mo *vs* 3.4 mo and 6.6 mo with regorafenib, respectively. Another supporting study was a retrospective analysis that investigated the feasibility and efficacy of oxaliplatin rechallenge in mCRC patients previously treated with adjuvant or palliative oxaliplatin-based chemotherapy, who had remained disease-free or progression-free for at least 6 mo after the last dose of oxaliplatin-based therapy[15]. Sixty-five patients were rechallenged with FOLFOX and 45 patients were rechallenged with XELOX. The median PFS and OS in this study with oxaliplatin rechallenge were 5.9 mo and 18.5 mo, respectively[15].

Fernandes *et al*[16] conducted a retrospective study to evaluate the benefit of rechallenging patients with mCRC to 5-fluorouracil, irinotecan, and oxaliplatin therapy (FOLFIRINOX or FOLFOXIRI). Twenty-one patients were retrospectively analyzed, with a response rate was 38% and 24% of patients had stable disease after rechallenge therapy. The median OS was 8.6 mo and only one patient had experienced grade 5 neutropenic sepsis. Another retrospective study had examined a South Australian mCRC database for patients who were rechallenged with FOX therapy (oxaliplatin and fluoropyrimidine)[17]. The study included 20 patients and discovered that for this patient cohort, the response rate was 18% and 48% of patients had stable disease after oxaliplatin rechallenge. The median PFS and OS were 3.7 and 7.8 mo, respectively[17]. Another study reported a comparable OS to rechallenge therapy, a 7-mo median OS, and 32% DCR[18]. This particular study investigated oxaliplatin-based chemotherapy rechallenge in mCRC patients previously treated with oxaliplatin, irinotecan, bevacizumab, cetuximab, or panitumumab therapies (if wild-type *KRAS*)[18]. Though these retrospective studies support the rationale of oxaliplatin rechallenge as another third/fourth-line option for mCRC, the concern with such studies is that there is no formal assessment as to whether oxaliplatin rechallenge leads to worsening

PSN in this patient cohort.

If considering chemotherapy rechallenge with an oxaliplatin-based regimen, clinicians should be wary of the development of PSN, which may lead to dose reduction of therapy, premature cessation of treatment, and significantly impair quality of life. To investigate whether oxaliplatin rechallenge results in new or worsening PSN, Besora *et al*[19] conducted a retrospective clinical study of 106 patients who were rechallenged with FOLFOX4/6, XELOX, or TOMOX. PSN was graded according to the National Cancer Institute-Common Toxicity Criteria for Adverse Events[20]. The study found that before oxaliplatin rechallenge, the frequencies of oxaliplatin-associated grade 1 and 2 PSN were 23.8% and 8.5%, respectively. After oxaliplatin rechallenge, 39.6% and 22.6% of patients developed grade 1 and 2 PSN, respectively; No patients developed grade 3 PSN. About 31% of all patients in this study experienced worsening PSN symptoms, whereas 68.9% of patients had the same PSN grade as before rechallenge therapy. This study sheds light on how oxaliplatin rechallenge may be a safe option, albeit neurological monitoring using scales such as the total neuropathy score, should be considered for mCRC patients who may undergo rechallenge therapy. Furthermore, a balance between rechallenge therapy to improve survival *vs* its impact on worsening PSN on quality of life for patients is crucial.

CONCLUSION

There are limited therapeutic options for mCRC that has progressed past the third and fourth lines of therapy. Rechallenging a chemo-resistant tumor with a previous well-tolerated and responsive line of chemotherapy may be a life-prolonging therapeutic approach for mCRC. This case demonstrates that rechallenge with IROX may offer a valid treatment option for mCRC patients with chemo-resistant disease, particularly in select patients with previous favorable response to oxaliplatin-based chemotherapy. IROX may serve as a viable option for rechallenge therapy, as seen in this case. However, neurological monitoring should be considered for mCRC patients who may undergo oxaliplatin-based rechallenge therapy. Further studies to elucidate the cellular mechanisms and predictive factors associated with enhanced response to rechallenge therapy in mCRC are warranted.

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