



ISSN 2220-7034 (online)

World Journal of *Medical Genetics*

World J Med Genet 2013 November 27; 3(4): 14-54
Volume End



 *Academic Publishing Group*
www.wjmg.net

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World Journal of *Medical Genetics*

World J Med Genet 2013 February 27; 3(1): 1-4





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1

Malignant pheochromocytoma in neurofibromatosis; mutation screening of RET proto-oncogene, *VHL* and *SDH* gene

Hasani-Ranjbar S, Amoli MM, Noorani M, Ghadami M

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

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Fax: +852-6555-7188
Telephone: +852-3177-9906
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PUBLICATION DATE
February 27, 2013

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Malignant pheochromocytoma in neurofibromatosis; mutation screening of RET proto-oncogene, *VHL* and *SDH* gene

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Author contributions: Hasani-Ranjbar S performed clinical and biochemical tests and followed up the patient, writing and editing the final article; Noorani M prepared the first draft of the paper and helped in writing the manuscript; Amoli MM performed genetic evaluation for von Hippel-Lindau and succinate dehydrogenase complex subunits; Ghadami M performed genetic tests for RET proto-oncogene and helped in writing the manuscript.

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Received: January 29, 2013 Revised: February 11, 2013

Accepted: February 20, 2013

Published online: February 27, 2013

Abstract

AIM: To investigate pathogenic mutations related to malignant pheochromocytoma in neurofibromatosis (NF).

METHODS: We present a patient with NF and metastatic pheochromocytoma in whom genetic screening for presence of pathogenic mutations in RET proto-oncogene, von Hippel-Lindau (*VHL*) and succinate dehydrogenase complex subunits B (*SDHB*) genes were investigated. RET proto-oncogene mutation screening for exons 10, 11, 13, 14, 15, 16 were examined by polymerase chain reaction (PCR) and direct DNA sequencing in patient. Mutation screening for exons 1, 2, 3 of *VHL* gene was carried out. Both forward and reverse strands

were subjected to direct sequencing after PCR amplification. The entire coding sequence of *SDHB* gene was screened for the presence of pathogenic mutations by PCR-sequencing.

RESULTS: A 45-year-old man presented with abdominal pain and hypertension over the previous year. The patient was a known case of neurofibromatosis type 1 (NF1) who presented at the age of 15 years with hyperpigmented and hypopigmented lesions. After complete evaluation for hypertension, biochemical tests and imagings indicated a malignant pheochromocytoma of 120 mm × 70 mm in size. The patient underwent left adrenalectomy, nephrectomy and splenectomy. After surgery the symptoms improved and blood pressure was controlled. After 5 years he was admitted again for evaluation of hypertensive crisis. Biochemical tests were again consistent with pheochromocytoma and disease relapse. Imaging studies and liver biopsy confirmed metastatic pheochromocytoma to the liver and para-aortic area. 131 Iodine-metaiodobenzylguanidine therapy was carried out. Genetic screening of *VHL* (exons 1, 2, 3), RET proto-oncogene (exons 10, 11, 13, 14, 15, 16) and *SDH* complex subunits revealed no pathogenic mutation.

CONCLUSION: We conclude that mutations in the *NF1* gene are responsible for the patient's clinical findings. However, would be helpful to further examine somatic mutations for a more precise study of genotype-phenotype correlation.

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Key words: Neurofibromatosis; Familial pheochromocytoma; Malignant pheochromocytoma; Metastatic pheochromocytoma; RET proto-oncogene; von Hippel-Lindau; Succinate dehydrogenase complex subunits

Core tip: Malignant pheochromocytoma associated with neurofibromatosis (NF) is very rare. We screened for all

possible mutations related to pheochromocytoma in a patient with NF and malignant pheochromocytoma but found no mutations. This negative result shows that the *NF1* gene is responsible for this rare presentation.

Hasani-Ranjbar S, Amoli MM, Noorani M, Ghadami M. Malignant pheochromocytoma in neurofibromatosis; mutation screening of RET proto-oncogene, *VHL* and *SDH* gene. *World J Med Genet* 2013; 3(1): 1-4 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i1/1.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i1.1>

INTRODUCTION

Pheochromocytomas and paragangliomas (PGLs) are catecholamine-secreting tumors, which arise from chromaffin cells of the adrenal medulla and extra-adrenal sites^[1]. Most pheochromocytomas are sporadic tumors. However some patients suffer from the disease as part of a familial disorder (15%-30%). There are several familial disorders associated with pheochromocytoma including von Hippel-Lindau (*VHL*) syndrome, multiple endocrine neoplasia type 2 (*MEN2*), neurofibromatosis type 1 (*NF1*), succinate dehydrogenase (*SDH*) complex subunit mutation-related tumors and occasionally *MEN1* syndrome. The approximate frequency of pheochromocytoma in these disorders is 50% in *MEN2*, 10%-20% in *VHL* syndrome, and 0.1%-5.7% with *NF1*^[2-5].

Malignant forms of catecholamine-secreting tumors are rare. The malignancy rate is variable from 2.4% to 26%. There are no histological proofs of malignancy for such tumors to date and the only accepted criterion is the presence of metastasis. The distant metastases are usually of hematologic origin, mostly involving bone, liver and lung^[2,3]. The prevalence of metastasis is up to 36%-50% for extra-adrenal abdominal pheochromocytoma and 10% and 5% for adrenal and familial forms respectively^[6,7]. However, in practice the diagnosis of malignant pheochromocytoma can only be determined by presence of recurrence or metastatic disease at a site where chromaffin cells do not normally exist. NF is the term given to two neurocutaneous genetic conditions. *NF1*, also known as von Recklinghausen's disease is the most common type of NF, with an incidence of approximately 1 in 2600 to 1 in 3000 individuals^[8]. Approximately half of the cases are familial while the remainder are new mutations^[9]. The hallmarks of *NF1* are the multiple café-au-lait spots and associated cutaneous neurofibromas. Pheochromocytoma has been clinically identified in 0.1%-5.7% of patients with NF. The *NF1* gene has been mapped to chromosome 17q11.2 and cloned^[10-12]. Since malignant pheochromocytoma is very rare in NF, we present a case with NF and metastatic pheochromocytoma in which genetic screening in *VHL*, Ret protooncogene and *SDH* were carried out to investigate the responsible genomic mutation.

MATERIALS AND METHODS

Biochemical testing and Localization Studies

Routine biochemical tests, evaluation of 24 h urine catecholamine metabolites, abdominal computed tomography or magnetic resonance imaging and 131 Iodine-metaiodobenzylguanidine (131I-MIBG) therapy were carried out. The malignant pheochromocytoma was diagnosed according to the presence or absence of metastasis in radiological or pathological reports.

Genetic analysis

RET proto-oncogene mutation screening: Genomic DNA was extracted from peripheral lymphocytes using the salting out technique. RET proto-oncogene mutation screening for exons 10, 11, 13, 14, 15, 16 was performed polymerase chain reaction (PCR) and direct DNA sequencing using the assay described by Alvandi *et al.*^[13].

VHL gene mutation screening: For *VHL* gene mutation screening, analysis of *VHL* gene was performed using the protocol of Cruz *et al.*^[2,14]. Exons 1, 2, 3 of *VHL* gene were amplified by PCR with the following primers: 1F - 5' CCATCCTCTACCGAGCGCGCG 3'; 1R - 5' GGGCTTCAGACCGTGCTATCG 2; 3F - 5' TGCCAGCCACCGGTGTG 2; 3R - 5' GTCTATCCTGTACTTACCACAACA; 3F - 5' CACACTGCCACATACATGCACTC 3'; 3R - 5' ACTCATCAGTACCATCAAAAGCTG 3'. Both forward and reverse strands were subjected to direct sequencing after PCR amplification^[2].

SDH complex subunits B gene mutation screening:

The entire coding sequence of the succinate dehydrogenase complex subunits B (*SDHB*) gene was screened for the presence of pathogenic mutations by PCR-sequencing based on the assay described previously^[15].

RESULTS

Patient

A 45-year-old man presented with abdominal pain and hypertension over the previous year. The patient was a known case of *NF1* who presented at the age of 15 years with hyperpigmented and hypopigmented lesions on the trunk, arms, feet and axillary areas. Family history was positive for NF in his mother and brother. Biochemical tests including urine metanephrines and imaging were compatible with malignant pheochromocytoma. In scans there was a large mass in the left adrenal with a size of 120 mm × 70 mm and invasion to left kidney was reported. The patient underwent left adrenalectomy, nephrectomy and splenectomy. After surgery the symptoms improved and blood pressure was controlled. The patient had poor compliance in follow up. After 5 years he was admitted again for evaluation of a hypertensive crisis. Biochemical tests were again consistent with pheochromocytoma and disease relapse. Imaging study and liver biopsy confirmed metastatic pheochromocytoma in the

liver and para-aortic area. 131I-MIBG therapy was carried out.

Genetic analysis

RET proto-oncogene mutation screening: There was no pathogenic mutation in the RET proto-oncogene. Only three single nucleotide polymorphisms were identified in this patient as follows: (1) C1866T in exon 10, causes no AA change (P622P); (2) G2071A in exon 11, causes G691S; and (3) C2712G in exon 15, causes no AA change (S904S).

VHL gene and SDHB gene mutation screening: There was no VHL gene mutation. In addition, genetic sequencing of 8 exons of SDHB gene revealed no pathogenic mutation in this patient.

DISCUSSION

The family described in this study comprises individuals with NF and one member (a 45-year-old man) with hypertension diagnosed as malignant pheochromocytoma. Hypertension is a frequent finding in adults with NF1 and may develop during childhood although pheochromocytoma is a much less common etiology. In these patients, the catecholamine-secreting tumor is usually a solitary benign adrenal pheochromocytoma, occasionally bilateral adrenal pheochromocytoma, and rarely a peri-adrenal abdominal PGL. Although NF1 as an autosomal dominant disorder is the most common familial cancer syndrome predisposing to pheochromocytoma, the risk of pheochromocytoma in this disorder is only about 1%^[16,17].

Pheochromocytomas in patients with NF1 occur in the fifth decade. Our patient was a 45-year-old man with an unusual presentation of pheochromocytoma. Currently, except for the presence of the SDHB mutation, a large or an extra-adrenal primary tumor, there are no reliable markers for predicting a high likelihood of developing metastatic disease. Pheochromocytoma in NF is usually benign and unilateral. Based on genetic background our expectation before surgery was a benign non-metastatic tumor. As mentioned before, the patient had a metastatic tumor and after 5 years metastasis to liver and para-aortic lymph nodes led to deterioration in the clinical course of disease. It seems to us that a large mass (as detected in this patient initially) as well as local invasion are critical factors for predicting malignancy and may have the highest impact for detecting metastasis in future.

At first we treated the patient with surgical resection of the adrenal mass, but in follow up treatment of distance metastasis the only available modality was MIBG therapy. Given the positive MIBG scan, we predicted that the tumor would up take iodine. To date, beside surgery, 131I-MIBG therapy is the single most valuable option for malignant pheochromocytomas. Results of a phase II trial using high dose 131I-MIBG demonstrated 22% partial or complete response and 35% of patients having some degree of response (*i.e.*, biochemical) without dem-

onstrated progressive disease^[18]. After 6 mo follow up our patient is alive but needs antihypertensive drugs. In future, our plan could be chemoembolization of the liver if there is persistent disease. Mutation screening were negative in our patient except for three nucleotide changes in the RET gene. Among them, nucleotide change C1866T is a new SNP, while changes G2071A and C2712G have been reported in the literature as SNPs. None of these changes are causative mutations for medullary thyroid cancer (MTC) patients although they are more common in patients with MTC than in normal population. Whether there is an interaction between these polymorphisms and other genes related to NF is still a matter of debate.

Mutations in the NF1 gene result in loss of functional protein, causing the wide spectrum of clinical findings with NF1-associated tumors. No obvious genotype-phenotype correlation between small mutations (< 20 base pairs) of the NF1 gene and a specific phenotype have been demonstrated, with the exception of the c.2970-2972 delAAT (p.M990del) mutation that is associated with a very mild phenotype in the majority of cases^[19]. Genetic testing for NF1 is available but is not routinely performed, as the diagnosis is made based upon clinical phenotype.

In addition, epigenetic factors, particularly DNA methylation, may also play essential roles in regulation of RET, VHL and NF1 pathways. A recent study has identified methylated RET in colorectal cancer^[20]. It has been observed that the aberrant methylation of RET correlates with decreased RET expression, whereas the restoration of RET in colorectal cancer cell lines results in apoptosis^[21]. Other studies have found epigenetic regulation of VHL or NF1 genes in various carcinomas^[22,23]. Therefore, epigenetic regulation of involved genes may also contribute to the pathogenesis of disease in this patient and this requires further investigations.

ACKNOWLEDGMENTS

We gratefully thank this family for consenting to the publication of this study.

COMMENTS

Background

Neurofibromatosis (NF), is the term given to two neurocutaneous genetic conditions. NF type 1 (NF1), also known as von Recklinghausen's is the most common type of NF.

Research frontiers

Hypertension is a frequent finding in adults with NF1 and may develop during childhood but pheochromocytoma is a much less common etiology. In these patients, the catecholamine-secreting tumor is usually a solitary benign adrenal pheochromocytoma, occasionally bilateral adrenal pheochromocytoma, and rarely a peri adrenal abdominal paraganglioma.

Innovations and breakthroughs

At first we treated the patient with surgical resection of the adrenal mass, but in follow up for treatment of distance metastasis the only available modality was metaiodobenzylguanidine therapy.

Applications

Genetic testing for NF1 is available but is not routinely performed, as the diagnosis is made based upon clinical phenotype.

Peer review

This is a relatively interesting manuscript of a rare case report on Pheochromocytoma in *NF1*. Although mutational studies of potential related genes were performed, the negative findings do not provide any additional molecular data of interest.

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Name of journal

World Journal of Medical Genetics

ISSN

ISSN 2220-3184 (online)

Launch date

December 27, 2011

Frequency

Quarterly

Editor-in-Chief

Hans van Bokhoven, Professor, PhD, Department of Human

Instructions to authors

Genetics and Cognitive Neurosciences, Radboud university Nijmegen
Medical centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands

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World Journal of Medical Genetics
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjmg@wjgnet.com
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Publisher

Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza, 315-321 Lockhart Road,
Wan Chai, Hong Kong, China
Telephone: +852-58042046
Fax: +852-31158812
E-mail: bpgoffice@wjgnet.com
<http://www.wjgnet.com>

Production center

Beijing Baishideng BioMed Scientific Co., Limited
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381892
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Instructions to authors

Full instructions are available online at http://www.wjgnet.com/2220-3184/g_info_20100722180909.htm.

Indexed and Abstracted in

Digital Object Identifier.

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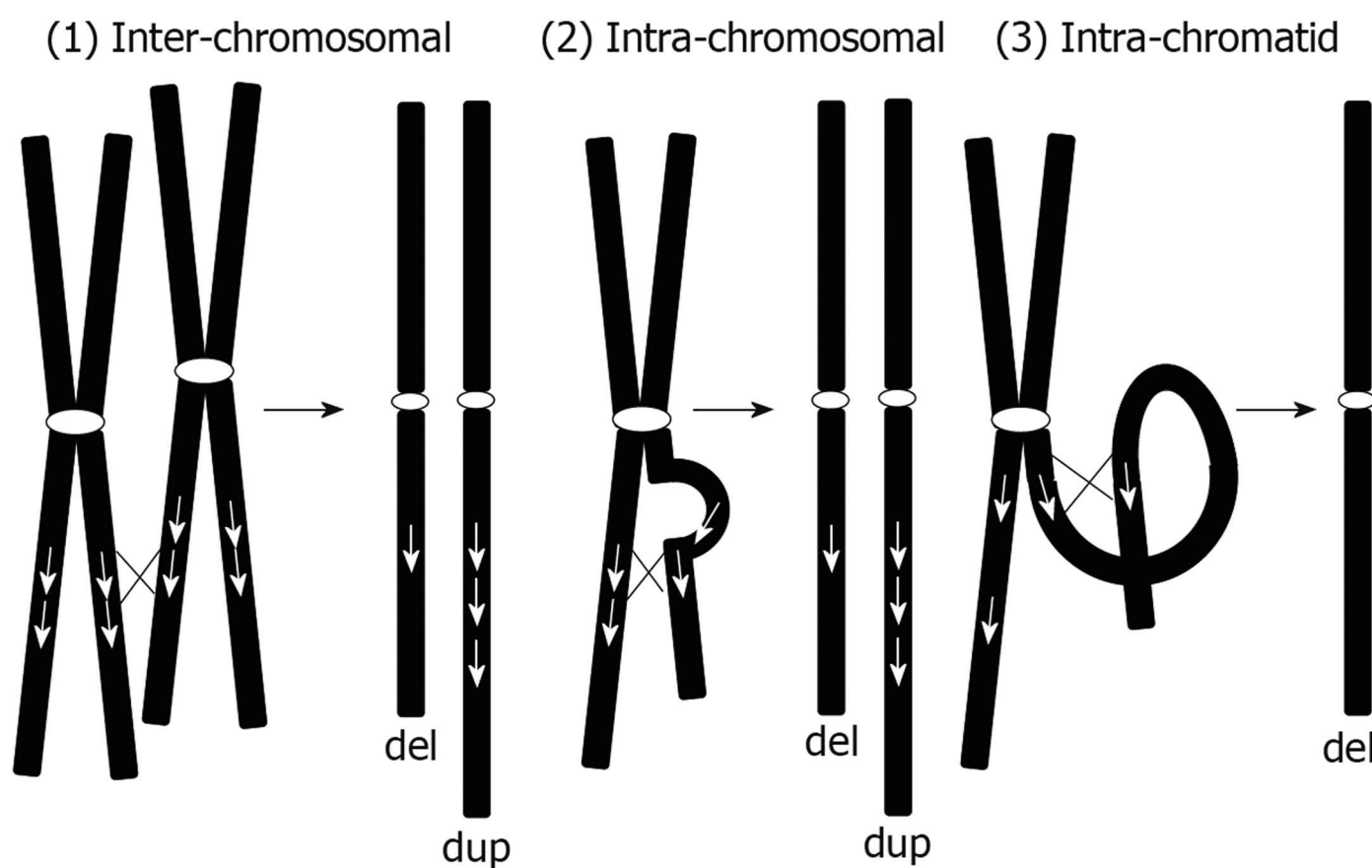
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5

Fiber-fluorescence *in situ* hybridization analyses as a diagnostic application
for orientation of microduplications

Yamamoto T, Shimada S, Shimojima K

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World Journal of Medical Genetics
Volume 3 Number 2 May 27, 2013

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

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Quarterly

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PUBLICATION DATE
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Fiber-fluorescence *in situ* hybridization analyses as a diagnostic application for orientation of microduplications

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Author contributions: Yamamoto T wrote the manuscript; Shimada S and Shimojima K collected the data.

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Received: March 23, 2013

Revised: May 14, 2013

Accepted: May 20, 2013

Published online: May 27, 2013

and triplicated segments of chromosomes. Previously, we reported that interstitial duplications were aligned in tandem configurations, supporting the hypothesized mechanism of non-allelic homologous recombination; however, there were rare cases of inverted duplications. Further analysis is therefore required to fully elucidate the basic mechanisms underlying such duplications/triplications.

Yamamoto T, Shimada S, Shimojima K. Fiber-fluorescence *in situ* hybridization analyses as a diagnostic application for orientation of microduplications. *World J Med Genet* 2013; 3(2): 5-8 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i2/5.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i2.5>

Abstract

Microduplications are normally invisible under microscopy and were not recognized before chromosomal microarray testing was available. Although it is difficult to confirm the orientation of duplicated segments by standard fluorescence *in situ* hybridization (FISH), our data indicates that fiber-FISH analysis has the potential to reveal the orientation of duplicated and triplicated segments of chromosomes. Recurrent microduplications reciprocal to microdeletions show tandem orientations of the duplicated segments, which is consistent with a non-allelic homologous recombination mechanism. Several random duplications showed tandem configurations and inverted duplications are rare. Further analysis is required to fully elucidate the basic mechanisms underlying such duplications/triplications.

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Key words: Chromosomal microarray testing; Copy number variation; Fiber-fluorescence *in situ* hybridization; Microduplication; Tandem orientation

Core tip: Fiber-fluorescence *in situ* hybridization analysis has the potential to reveal the orientation of duplicated

COMMENTARY ON HOT TOPICS

Background

Chromosomal microduplication is a type of chromosomal anomaly. Although several chromosomal duplications have been reported previously, small microduplications that were invisible under the microscope were not recognized before chromosomal microarray testing was available. The only known microduplications were those involving specific genes, such as the peripheral myelin protein 22 gene (*PMP22*) and the proteolipid protein 1 gene (*PLP1*). Previously, we knew that patients with *PMP22* mutations manifest Charcot-Marie-Tooth disease [Mendelian Inheritance in Man (MIM) #118220] and patients with *PLP1* mutations show Pelizaeus-Merzbacher disease (PMD; MIM #312080); microduplications of these genes were revealed under the hypothesis that copy number gain of these genes may be related to disease occurrence^[1]. Before the availability of chromosomal microarray testing, a targeting system was used to detect such duplications, including fluorescence *in situ* hybridization (FISH), quantitative polymerase chain reaction (PCR) and multiple ligation probe amplification (MLPA) (Table 1).

It is possible to detect the numbers of the targeted signals by FISH - whole subtelomeres can be analyzed in this manner^[2]. Specific regions have already been analyzed previously using this method; *e.g.*, the 22q11.2 region for DiGeorge syndrome and the 7q11.2 region for Williams syndrome. Although FISH is a powerful tool for detecting deletions of targeted regions, the biggest disadvantage is that it is difficult to detect small duplications owing to overlapping signals.

Generally, PCR cannot be used to quantify amplified fragments owing to saturation of amplification. However, in cases of linear amplification, quantity can be estimated by using a real-time PCR monitoring system. The chief feature of this system is that the target is fixed at the start of analysis^[3]. Although MLPA is based on a PCR system, the targets of amplification are fused probes included in the buffer. Because various sizes of the amplicons can be included in the same tube, multiplexed targets can be measured simultaneously^[4] and whole subtelomeric regions can be analyzed comprehensively^[5].

At present, it is easier to detect genomic copy number aberrations by using chromosomal microarray testing as a comprehensive method. We subsequently realized that there are numerous microchromosomal duplications that are genetic causes for various diseases.

Mechanism of microduplications

We can classify duplications into 2 types: recurrent microduplications and random microduplications (Table 2). Recurrent microduplications are caused by non-allelic homologous recombination (NAHR) mediated by low-copy repeats. Consequently, such microduplications are reciprocal to the microdeletions caused by the NAHR mechanism. According to this mechanism, chromosomal aberrations can occur in 3 ways^[6]: (1) inter-chromosomal; (2) intra-chromosomal; and (3) intra-chromatid (Figure 1). As shown in Figure 1, inter-chromosomal and intra-chromosomal exchanges can create both deletions and duplications equally. However, intra-chromatid exchange only creates microdeletion and not microduplication. Thus, microduplications created by NAHR are definitely a consequence of inter-chromosomal or intra-chromosomal exchange. Both processes create duplications in tandem orientation. However, few studies have confirmed the hypothesized tandem configurations of the duplication.

Standard FISH

Previously, many efforts have been made to detect orientations of the duplication by FISH. The combined use of 2 different colored probes can detect the orientations of the duplicated segments. We analyzed the orientations of the duplicated segments and many showed tandem configurations. As shown in Figure 2A, an interstitial duplication of 2q32.1-q33.3 was inserted in a contiguous tandem configuration^[7].

Compared to such interstitial duplications, subtelomere duplications are different because they are most commonly a result of U-type exchange between the sister chromatids during the meiotic process^[8]. We observed

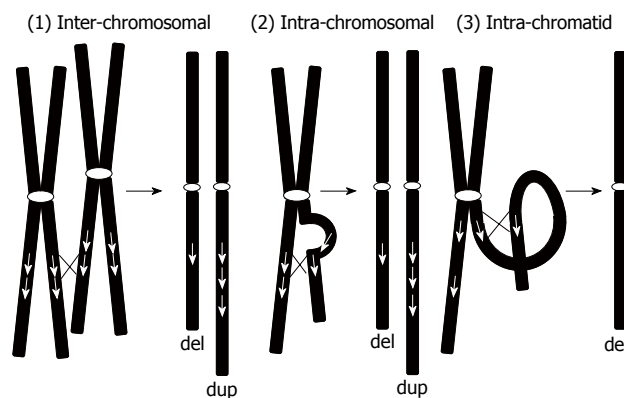


Figure 1 Schematic representation of the mechanism of non-allelic homologous recombination. The mechanism can occur in 3 ways. Chromosomal duplications can be created by (1) inter-chromosomal exchange; (2) intra-chromosomal exchange; and (3) intra-chromatid exchange. Although inter-chromosomal and intra-chromosomal exchanges can lead to deletions and duplications equally, intra-chromatid exchange only creates microdeletions and not microduplications. Microduplications created by non-allelic homologous recombination invariably show tandem orientations. White arrows indicate the directions of the segments. This figure is referring the report by Gu *et al.*^[6].

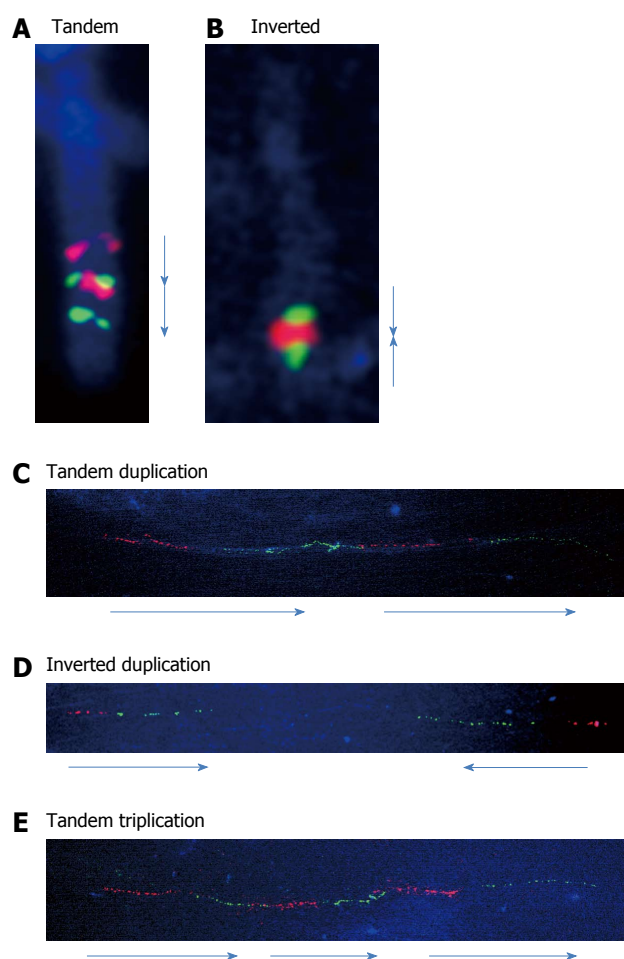


Figure 2 Results of fluorescence *in situ* hybridization in metaphase and fiber-fluorescence *in situ* hybridization. A: Tandem duplication aligned in red-green-red-green is shown; B: Inverted duplication aligned in green-red-red-green is shown; C: Tandem duplication aligned in red-green-red-green is shown in a single DNA fiber; D: Inverted duplication aligned in red-green-green-red is shown; E: Tandem triplication with 3 repeats of red-green units. Arrows indicate the directions of the segments.

Table 1 Characters of the methods to detect genomic copy number variations

Characters	Advantages	Disadvantages
Fluorescence <i>in situ</i> hybridization	Numbers of the signals correspond to the genomic copy numbers	Small duplications cannot be detected
Quantitative polymerase chain reaction	Wide dynamic range Many samples can be analyzed at once	Primer designs are required for each targets
Multiple ligation probe amplification	Multiple targets can be analyzed at once	Original primer designs are required for specific regions
Chromosomal microarray testing	Accurate and high resolution Comprehensive	Chromosomal structures including balanced translocations cannot be analyzed

Table 2 Characteristics of microduplications

Characteristic	Recurrent microduplications	Random microduplications
Mechanism	Non-allelic homologous recombination Mediated by locus control regions	Random
Characteristics	Reciprocal to common deletions Uniformed size	Random

inverted duplication and deletion in the 10q26 region^[9]. Inverted orientation was confirmed by FISH in this case (Figure 2B).

As mentioned above, in the cases of large duplications it is possible to determine the orientations of the duplicated segments; however, it is difficult to determine orientation for small duplications owing to overlapping signals in metaphase spreads. Consequently, interphase nuclei were used as an alternative to determine the alignments of the signals. Lee *et al.*^[10] analyzed the orientations of the duplications involving *PLP1* in patients with PMD using interphase nuclei. They confirmed that many duplications of this region were aligned in tandem orientations. However, there is the limitation that directions cannot be detected accurately. To compensate for this, several nuclei from the same patients have to be checked.

Methods of fiber-FISH

Chromosomes are composed of 3-dimensional structures that consist of DNA and histones. Thus, targeted signals can be overlapped. In such cases, fiber-FISH analysis has the advantage of being able to detect the orientations of the duplicated segments accurately^[11-13] ("fiber" means DNA fibers).

DNA fiber specimens can be prepared after separating chromatin structures by surfactants. To perform fiber-FISH analysis, traditional Carnoy fixation can be used. The method is as follows: Carnoy-fixed samples should be mounted on the surface of the glass slides and the slides should be immediately dipped into sodium dodecyl sulfate solution and then slowly pulled out. Consequently, the separated DNA fibers can be fixed onto the surface of the glass slides.

The same probes used for standard FISH can be used for fiber-FISH. Compared to standard FISH, targeted signal intensity is extremely weak. Because live signals are not visible by microscopy, long exposure time is required

for signal capture. To capture standard FISH pictures, interphase nucleus or metaphase labeled by DAPI can be used as landmarks; however, there are no interphase nucleus and metaphase in fiber-FISH specimens. Thus, it is necessary to search the entire surface of the slide to detect signals.

Not all fibers show the same rate of extension on the same slide. This is dependent on the duration of the dip into the surfactants solution. If the length of the targeted fiber was too long to be captured in a single field of view, not all the target signals can be captured in the same field of view. It also depends on the size of the targeted duplications. If signals can be successfully captured, the directions of the fragments can be determined.

In terms of the clinical point of view, detection of microduplication orientation is not a suitable strategy for clinical analyses. Thus, fiber-FISH analysis is a specialized method for research work.

Orientations of the duplicated segments

Previously, we analyzed the directions of the duplicated segments of the 22q11.2 region. The 4 duplications of the 22q11.2 region, identified by chromosomal microarray testing, showed tandem configurations of the duplicated segments, supporting the hypothesized mechanism of NAHR^[11]. We analyzed 7 and 4 samples with the duplications in the region of *PLP1* and the methyl CpG binding protein 2 gene, respectively^[12-14], and all of the duplications showed tandem configurations as seen in the 22q11.2 region (Figure 2C), although the duplications in the region of *PLP1* are considered to be created by fork stalling and template switching and not by NAHR^[15].

Almost all duplications analyzed previously showed tandem configurations, as seen in cases of large duplications visible by general FISH analysis. The only exception was a benign copy number gain identified at the Xp22.31 region that included the steroid sulfatase gene (Figure 2D)^[12]. This may indicate that the mechanism of the copy number gain in the benign region may be different from that of the pathological CNV.

Triplications

Triplications are rare chromosomal aberrations which can be classified into 2 types: (1) triplication embedded into duplicated segments; and (2) triplications not embedded into duplicated segments^[16]. Because sufficient data are not available owing to the scarcity of these triplications,

the mechanisms underlying them have seldom been analyzed. The clearest evidence is that some of the triplications embedded into duplicated segments are caused by the duplication-inverted triplication-duplication mechanism revealed by Carvalho *et al.*^[17] and Shimojima *et al.*^[18]. Compared to this, a tandem triplication was confirmed in another case involving the platelet-activating factor acetylhydrolase 1b regulatory subunit gene (Figure 2E)^[19].

In conclusion, as a result of the wider adoption of chromosomal microarray testing as a diagnostic tool, many genomic copy number gains were found to cause multiple congenital anomalies and intellectual impairments. Thus, this is just a starting point to understanding the mechanism of such genomic copy number gains. For this purpose, we should accumulate more cases of duplications and triplications and analyze the orientations of the segments.

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- P- Reviewers** Corrales FJ, Li CJ, Petmitr S, Sipos F, Sterlacci W, Tanabe S **S- Editor** Gou SX **L- Editor** Roemmele A **E- Editor** Zheng XM



World Journal of *Medical Genetics*

World J Med Genet 2013 August 27; 3(3): 9-13





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World Journal of Medical Genetics
Volume 3 Number 3 August 27, 2013

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World Journal of Medical Genetics (*World J Med Genet*, *WJMG*, online ISSN 2220-3184, DOI: 10.5496) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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INDEXING/ABSTRACTING

World Journal of Medical Genetics is now indexed in Digital Object Identifier.

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

FREQUENCY
Quarterly

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Telephone: +86-10-85381891
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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Fax: +852-6555-7188
Telephone: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
<http://www.wjgnet.com>

PUBLICATION DATE
August 27, 2013

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Genetic counselling in post-genomic era-to be or not to be

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Received: June 3, 2013 Revised: July 27, 2013

Accepted: August 17, 2013

Published online: August 27, 2013

consumer genetic testing; Clinicians and public health policy makers

Core tip: This paper aims at discussing the aspects and challenges which have to be faced during genetic counselling in the new post-Human Genome Project era with beneficial impact on human disease, health care, and medical benefits. With the surge of genetic tests and technologies, genetic counsellors are faced with the challenge of translating emerging scientific knowledge into practical information for patients, clinicians same as for public health policy makers and the needs for genetic counselling should be designed into genomic research at the onset. Genetic counsellors need to handle old while rapidly assimilating new information.

Abstract

With the surge of genetic tests and technologies, genetic counsellors are faced with the challenge of translating emerging scientific knowledge into practical information for patients, clinicians and public health policy makers. The new tests and technologies also are associated with new psychosocial and ethical considerations. New guidelines are needed for each new discovery of the genomic impact on phenotype, pathology and disease while "old" syndromes and "old" pathology, continue to require attention. In the new post-Human Genome Project era, genetic counsellors will be an integral part of translating genomic discoveries into beneficial impact on human disease, health care, and medical benefits. The needs for genetic counselling should be designed into genomic research at the onset. Genetic counsellors need to handle old while rapidly assimilating new information and the principal challenge is to be up to date and updated.

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Key words: Genetic counseling; Clinical application; Translating emerging scientific knowledge; Direct-to-

Nenad B, Maurizio M. Genetic counselling in post-genomic era-to be or not to be. *World J Med Genet* 2013; 3(3): 9-13 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i3/9.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i3.9>

INFLUENCE ON GENETIC COUNSELLING/ COUNSELLORS AND OTHER HEALTH PROFESSIONALS

The spectacular progress in understanding the genetic nature of disease has deeply changed the daily practice of medicine. With the surge of genetic tests and technologies, genetic counsellors are faced with the challenge of translating emerging scientific knowledge into practical information for patients, clinicians and public health policy makers. The new tests and technologies also are associated with new psychosocial and ethical considerations. As reported by Bennett *et al*^[1], the field of genetic counselling arose from the need to educate, manage and counsel individuals and their families diagnosed with, or at risk for, genetic diseases with respect to how these conditions may affect the psychological, medical, finan-

cial and social aspects of one's life.

Genetic counselling is now a necessary component of the practice of virtually all medical specialties. Physicians must help their patients understand a genetic diagnosis and assist them in making and coping with decisions relating to the diagnosis. As each new genetic test is made available in the clinic, developing the appropriate counselling for each new diagnosis is necessarily a multidisciplinary endeavour that includes involvement of a Specialist of Medical Genetics^[2,3]. This paper aims at discussing the aspects and challenges which have to be faced during genetic counselling.

WHAT'S NEW?

The Human Genome Project provide us not only with information regarding the basic architecture of human genome, it also gave rise to impressive advances in molecular technologies. It is now possible to routinely assess genetic variation at a population level. For example, it is routine to assess over a million single nucleotide polymorphisms (SNPs) on thousands of individuals within a single study and it is routine to combine studies into meta-analyses across hundreds of thousands of individuals^[4]. An excellent review of Gao *et al.*^[4] discussed the use of genome-wide association studies (GWAS). This strategy is based upon the common disease common variant hypothesis^[5], in which it is proposed that high-prevalence traits are determined by high-frequency genetic variants. Some successful examples are given by meta-analyses in GWAS in Parkinson's disease^[6], type 2 diabetes^[7,8], type 1 diabetes^[9], chronic kidney disease^[10], retinal microcirculation^[11], Crohn's disease^[12], and others. Beyond simply examining nucleotide variations, new technology allows researchers to assess other aspects of genomic variations including whole transcriptome profiling and genome-wide epigenetic modifications. Now the major challenge in genomics is to apply this rapidly expanding plethora of genetic data in meaningful ways to further improve our understanding of human biology^[13] and to generate knowledge about the genetic contribution to human diseases^[14].

While research focuses on how to put the human genome in context, it should not be forgotten that it is quite "tricky" to translate these research data into appropriate genetic counselling of each client. Especially if we consider that the next step in personal genomics is to associate an individual's specific variation with clinical disease phenotypes, counselling must help individuals discriminate between medically important variation and benign polymorphic variation. Data of genomic variations must be carefully translated by a genetic counsellor with care to educate the clients of the presumed significance of genes and mutations, imprinting, and the likelihood of benign versus causative genomic changes^[15,16]. This means that genetic counsellors are at the forefront of introducing and applying the advances from genome

science to the lives of individuals and their families, by translating the complex language of genomic medicine into terms that are easy to understand^[1].

WHAT HAS BEEN CHANGED AS CONSEQUENCE OF NEW DIAGNOSTIC APPROACHES?

The era of genomic medicine challenges traditional definitions of "healthy" and "diseased". Traditionally, medical attention is only sought regarding a present illness. Now genetic testing permits the diagnosis of healthy individuals who are expected to develop or have an increased susceptibility to develop a disorder^[17,18]. Testing for susceptibility genes will push into the world of medicine millions of individuals who have no personal experience of any disease, as emphasized by Professor Dallapiccola^[19]. Some of them will benefit from the information, but many will become "unpatients", *i.e.*, individuals who are neither patients under treatment nor healthy individuals free of any medically relevant condition. This new class of individuals (it seems appropriate to call them "clients") who are watching and waiting for a sign of disease must be advised to undertake appropriate systematic clinical and instrumental monitoring while avoiding the development of psychosomatic symptoms. It is thus necessary to rethink the genetics revolution in medicine in terms of benefits and harm considering that the general rule for all physicians is: "*Primum non nocere*" ("First, do no harm"). After all, when we think about applications of genetics in daily practice, genetic counselling included, we should take in mind, J. Watson's observation: "I have benefited a lot from being the first human to have his or her personal genome made publicly available on the web. So far, knowledge of my personal genetic risks has not cost me an hour of sleep. I doubt, however, whether I would feel so positively if this knowledge had been given to me at a much earlier stage of my life"^[16,20].

It is necessary to introduce into genomic research consideration of computational strategies which permit translation of genetic information into clinically useful probability estimation. Personalized cancer risk assessment is an example of this integration. Algorithms in conjunction with testing have been successfully applied to predict the probability to carrier germinal at-risk mutations, as BRCAPRO for breast and ovarian cancer syndromes^[21-24], PancPRO for familial pancreatic cancer^[25] and MelaPRO for melanoma families^[26], *etc.*

ITALIAN EXPERIENCE

The Italian genetic testing survey 2004^[27], could be seen as starting point (at least for us who are working in Italy) for understanding the necessity to link testing and genetic counselling in order to cut the costs, and to widen the number of available services. This survey also stressed the necessity for constant training of the general practi-

tioner and education of the consumer with regard to the appropriate use of genetic tests. A more sparing use of genetic tests, which should always follow specific clinical indications, ideally flank and sustain good clinical practice. Conversely, inappropriate genetics testing do harm by imparting a false sense of reassurance in individuals found not to have a gene mutation who are not informed of the limitation of tests and are major contributors to increasing health care costs^[28,29].

“DO-IT-YOURSELF” GENOMIC TESTING

Direct-to-consumer genetic testing (DTC-GT) provides personalized genetic risk information directly to consumers. DTC-GT has generated a considerable controversy about its potential benefit, harms, and regulatory status since its entry into the mainstream marketplace in 2006^[30,31] largely as a result of the unclear link between DTC-GT results, consumer risk and cost effective health care decisions.

With DTC-GT, clients without the guidance of genetic counselling will often purchase a genetic test that is not clinically indicated. Individuals ordering and interpreting genetic tests for tens or hundreds of conditions with varying clinical validity and utility, in the absence of a healthcare professional, could lead to unnecessary or incorrect healthcare decisions or emotional distress in the clients^[32]. Furthermore afterwards clients may communicate the results to health-care professionals—it is left to these professionals to discuss the testing guidelines and clinical/diagnostic protocols and the usefulness and significance of the results, opening the door to distrust and misunderstandings if the test results are discounted^[33].

Obviously access to DTC-GT can be seen as a right for consumers to purchase the offered product and services. However, the open issues about whether and how to regulate the new heterogeneous DTC-GT industry should be systematically and carefully studied to ascertain the clinical utility, referral patterns and downstream costs^[32,33].

SOME PERSPECTIVES WHICH ARE BECOMING REALITY, IS THIS SCIENCE OR SCIENCE FICTION?

Autism spectrum disorders (ASDs) are an example of an emerging area for genomic diagnosis that will require parallel development of genetic counselling. ASDs are a heterogeneous group of neurodevelopmental disorders affecting social communication, language and behavior.

There have been reports of applying panels of common SNPs to assess ASD risk^[34,35], but these approaches require more testing/investigation before SNPs can be associated with risk. With rapid emergence of whole-genome sequencing studies, there will be an explosion of new data leading to more comprehensive genotype and phenotype studies^[36-38]. In addition to seeking to identify

genes that influence diseases, scientists are looking for genes which influence biological markers of disease or endophenotype. One example of this approach is the emerging field of imaging genomics which discover important variants associated with brain structure and function. In these studies, a high degree of correlation has been observed between genome and image-derived maps giving some explanation on how these variations influence disease risk and fundamental cognitive processes^[39].

Parents of children with ASDs are generally aware that their subsequent children are at increased risk to be ‘on the spectrum’, but parents often over- or underestimate this risk. While the studies to date indicate that it may be possible, as yet no definitive genomic diagnostic or prognostic indicators of ASD have been found that can be used for risk estimation. The genetic testing and counselling approach to individuals with ASDs will continue to evolve as we learn more about the genetic factors involved and their relative contributions. The next step is to interpret this data and translate it in comprehensive and useful genetic counselling.

LAST BUT NOT LEAST

The scope of genomic counselling are growing rapidly. New guidelines are needed for each new discovery of the genomic impact on phenotype, pathology and disease while “old” syndromes and “old” pathology, for example Down Syndrome, continue to require attention. That is one of the reasons why the guidelines such as those published by National Society of Genetic Counsellors-Sheets *et al*^[40], will be always welcomed and “evergreen”.

In the new post-Human Genome Project era, genetic counsellors will be an integral part of translating genomic discoveries into beneficial impact on human disease, health care, and medical benefits. The needs for genetic counselling should be designed into genomic research at the onset. Genetic counsellors need to handle old while rapidly assimilating new information. The principal challenge is to be up to date and updated.

ACKNOWLEDGMENTS

The authors would like to thank John Elling, PhD, for his useful and constructive comments and suggestions on the manuscript.

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World Journal of *Medical Genetics*

World J Med Genet 2013 November 27; 3(4): 14-54

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AIM AND SCOPE *World Journal of Medical Genetics* (*World J Med Genet*, *WJMG*, online ISSN 2220-3184, DOI: 10.5496) is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

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INDEXING/ABSTRACTING *World Journal of Medical Genetics* is now indexed in Digital Object Identifier.

FLYLEAF I-IV Editorial Board

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NAME OF JOURNAL
World Journal of Medical Genetics

ISSN
ISSN 2220-3184 (online)

LAUNCH DATE
December 27, 2011

FREQUENCY
Quarterly

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PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza,
315-321 Lockhart Road, Wan Chai,
Hong Kong, China
Fax: +852-6555-7188
Telephone: +852-3177-9906
E-mail: bpgoffice@wjgnet.com
<http://www.wjgnet.com>

PUBLICATION DATE
November 27, 2013

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Genomic microarrays in prenatal diagnosis

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Received: May 8, 2013 Revised: July 20, 2013

Accepted: August 8, 2013

Published online: November 27, 2013

Abstract

The application of microarray-based techniques for the diagnosis of genomic rearrangements has been steadily growing in popularity since its introduction in 2004. Given the many advantages of these techniques over conventional cytogenetics, there is increasing pressure towards their application in prenatal diagnosis. However, there remain several important issues that must be addressed. For example, microarray-based techniques (comparative genomic hybridization-based arrays and single nucleotide polymorphism-based arrays) allow detection of even very small genomic imbalances that can determine pathological clinical conditions. In addition, there are other copy number variations which represent normal variation, with no detectable effects on phenotype. Given the still incomplete knowledge of the changes in our genome and the associated phenotypes, microarray-based diagnosis is likely to find variants of uncertain and unknown clinical significance. The interpretation of these variants is now a major challenge for the medical geneticist, who often find it difficult to establish precise correlations between genotype and phenotype. There is sufficient available evidence to justify the use of microarray-based diagnostics for a select number of specific conditions, but there is also an inevitable trend towards ever wider application. It is very important that this drift does not progress in an unchecked and uncontrolled manner under the thrust

of commercial interests. Therefore, we recommend that scientific societies be vigilant and take an advisory role in the adopting of these technologies as new scientific knowledge becomes available.

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Key words: Copy number variations; Genomic microarray analysis; Prenatal diagnosis

Core tip: Given its advantages over conventional karyotyping, there is an increasing interest in determining whether microarray technology will be similarly advantageous for the detection of fetal genomic imbalances in a prenatal setting. Several issues remain to be addressed, such as for which pregnancies comparative genomic hybridization-based arrays should be carried out (*i.e.*, whether for all pregnancies or only for those with ultrasound abnormalities). Another area of uncertainty is the choice of array platform. This article aims to contribute to the discussions on genomic microarrays in prenatal diagnosis by examining the literature and existing guidelines, and giving an opinion on possible future developments and on how best to handle them.

Lonardo F. Genomic microarrays in prenatal diagnosis. *World J Med Genet* 2013; 3(4): 14-21 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i4/14.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i4.14>

INTRODUCTION

The objective of prenatal diagnosis (PD) is to provide prenatal diagnostic testing services for genetic conditions that enable families to make informed choices consistent with their individual needs and values, and to support them in deal with the outcome of such testing.

PD is offered with the intention of determining the presence or absence of a pathological condition in the fe-

tus. Prenatal tests may be performed using invasive (such as amniocentesis, chorionic villus sampling or fetal blood sampling) or non-invasive procedures (such as analysis of cell-free fetal DNA in maternal blood or fetal imaging). PD is mainly offered to pregnant women in one of the following four groups: (1) Advanced maternal age; (2) Women and/or partners from families known to genetic or other relevant specialist services before pregnancy because of significant family history of a condition; (3) Women who are identified during pregnancy as having a fetus at risk of a genetic condition (for example, through disclosure of family history, possibly including genetic test results, during an antenatal consultation or following positive prenatal screening); and (4) Women whose fetus is identified as at risk of a genetic condition due to abnormal ultrasound findings.

As chromosomal anomalies are a major cause of perinatal morbidity and mortality, as well as the most frequent cause of intellectual disability in our population, cytogenetic diagnosis using cultured cells obtained by prenatal invasive tests has been regarded as the standard method for PD since its first application. In 1966, Steele *et al*^[1] reported the feasibility of performing chromosomal analysis of amniotic fluid cells. One year later Jacobson *et al*^[2] performed the first PD of a chromosomal abnormality (a balanced translocation), shortly followed by the first PD of trisomy 21^[3].

Karyotyping has proven highly reliable for the genome-wide detection of numerical chromosome abnormalities (aneuploidies) and large structural rearrangements in fetal cells. However, chromosome analysis has some important limitations. It takes about 15 d to culture the cells, visualize the chromosomes and perform the analysis, thus leading to anxiety in the pregnant women. The resolution of a karyotype is limited and chromosomal anomalies in the grey zone (between 5 and 10 Mb in size) lead to interpretation difficulties. Karyotyping also requires skilled analysts, which increases costs and can lead to organizational difficulties in small laboratories^[4].

More recently, molecular cytogenetic methods including interphase fluorescence *in situ* hybridization (FISH), quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) have been introduced for the rapid detection of aneuploidies of chromosomes 13, 18, 21 and sex chromosomes. These techniques can provide a result in 1-3 d but are disadvantaged by the need to perform locus-specific analysis, leaving a residual risk for a clinically significant chromosomal abnormality. In addition to the common aneuploidies, many submicroscopic chromosomal rearrangements that lead to copy-number gains or losses have been shown to cause distinct and recognizable clinical phenotypes.

The sensitivity in detecting copy-number alterations has increased significantly with the advent of genomic microarray analysis (GMA). Together with improved assemblies and annotation of genome sequence data, these methods allow rapid identification of new syndromes

that are associated with submicroscopic genomic changes in children with idiopathic intellectual disabilities (ID), autism, developmental delay (DD) and/or multiple congenital anomalies (MCA)^[5].

Genomic microarrays detect gains and losses of genomic regions through the hybridization of fluorescently labeled patient DNA onto targets with known genomic coordinates, spotted onto a solid substrate (typically a glass slide). By measuring the signal intensity ratio of patient DNA to a reference sample, gains or losses of genomic material can be identified.

Comparative genomic hybridization-based arrays (CGH-arrays) involve hybridization of a patient's DNA onto predetermined targets representative of the whole genome or of target regions [bacterial artificial chromosomes (BAC) clones of 100-200 kb or synthetic oligonucleotide probes of 25-75 bp] spotted onto glass slides. The patient DNA is extracted from the relevant sample, labeled with a fluorochrome, mixed with a reference DNA pool (labeled with a different fluorochrome) and then hybridized on the microarray slide^[6].

Single nucleotide polymorphism-based arrays (SNP-arrays) were originally designed to detect common SNPs (> 1% in the population) and were mainly used in genotyping individuals for genome-wide association studies of many common multifactorial diseases. In addition to SNP typing, these platforms can also be used to perform copy number analysis. Gains and losses of genomic regions can therefore be detected as is the case for CGH-arrays. SNP arrays also detect copy neutral loss of heterozygosity (or absence of heterozygosity), uniparental disomy and regions identical by descent. However, although SNP arrays detect uniparental isodisomy, parental samples are required for the detection of uniparental heterodisomy. When using SNP-arrays, only a single hybridization is performed for the patient DNA (single channel or color) and the signal intensities are then compared with a reference dataset^[7].

Microarray technology has several advantages over conventional karyotyping, including improved resolution and potentially higher detection rates of chromosomal variation. Using arrays, an additional 15% of causally related chromosomal abnormalities are detected over routine microscopic and MLPA or FISH for subtelomeric screening in patients with DD and/or MCA^[8]. In another study performed in postnatal patients (children and adults) with a diagnosis of unexplained neurodevelopmental disability, the positive diagnostic yield of CGH-array has been reported to be about 10% higher than that of standard karyotyping^[9].

In addition to providing higher resolution, the genomic microarray offers other potential advantages over conventional karyotyping, such as automation (and thus faster turnaround times) and elimination of the need to culture amniocytes or chorionic villi. Because microarray analysis does not require dividing cells, it is also useful in cases of fetal death, when it is often not possible to culture cells^[10]. Given the advantages of microarray-based technologies over karyotyping, there is increasing

Table 1 Data from the literature

Ref.	Key results
[11]	Array CGH could detect causative CNVs in children with ID, and other disabilities and congenital malformations
[12]	Feasibility of performing CGH-array for prenatal diagnosis on DNA extracted from AF cells
[13]	Evaluation of the literature up to 2009. Pathogenic CNVs or VOUS were detected in 3.6% of cases with a normal karyotype. Microarrays detected an additional 5.2% pathological CNVs or VOUS in pregnancies with fetal anomaly on ultrasound
[14-21]	Diagnostic utility of CGH- and SNP-arrays in a prenatal setting
[22]	Cohort studies, published from 2009 onwards, have demonstrated an increased detection rate over standard karyotyping ranging from 0.9% to 26.5%
[23]	Evaluation of the utility of a 1-Mb BAC and 60-K oligonucleotide array in 3171 pregnancies. The detection rate was low (0.52%) in uneventful pregnancies, but increased to 8.2% when a fetus had an abnormality on ultrasound scan
[24]	Additional information in 7.7% of cases using a SNP-array with a resolution of 150/200 kb to analyze DNA from 207 cases with fetal anomalies
[25]	Comparison of microarray with standard karyotyping in 4406 women undergoing PD for common indications over a period of 3 yr (2008-2011) The analysis identified all of the common autosomal and sex-chromosome aneuploidies and the unbalanced rearrangements detected by standard karyotyping in the 4282 non mosaic samples. Microdeletions or duplications of clinical significance were found in 96 of 3822 fetal samples with normal karyotypes (2.5%), including 6.0% of cases in which fetal anomalies were detected on ultrasonography. There were 94 copy-number variants of uncertain clinical significance that required further evaluation. The pathogenicity of 1.5% of CNVs remained uncertain
[26]	Exploration of the utility of microarray analysis in groups of pregnancies with a priori low risk for detection of submicroscopic chromosome abnormalities. A total of 3000 prenatal samples were processed in parallel using both microarray and conventional karyotyping. Samples were processed using a BAC platform with a resolution of about 1 Mb across the genome and about 100 kb in 139 regions associated with constitutional disorders. The percentage of detection was 0.5% (6/1118) in advanced maternal age and 0.7% (11/1674) in parental anxiety. No genetic imbalances were detected in any of the cases sampled for an abnormal maternal serum screening, nor for a family history of a genetic condition or chromosomal abnormality. A total of 24 (0.8%) fetal conditions would have remained undiagnosed if only a standard karyotype had been performed. 17 (0.6%) of such findings would have otherwise been overlooked if CMA was offered only to high risk pregnancies
[27]	Study on 5003 prospective cases received for a variety of indications. The overall detection rate of clinically significant CNAs was 5.3%. Detection rates were 6.5% and 8.2% for cases referred with abnormal ultrasounds and fetal demise, respectively. The overall rate of findings with VOUS was 4.2% but would reduce to 0.39% if only de novo CNAs were considered. In cases with known chromosomal rearrangements in the fetus or parent, 41.1% showed CNAs related to the rearrangements, whereas 1.3% showed clinically significant CNAs unrelated to the karyotype. 71% of the clinically significant CNAs found by microarray were below the resolution of conventional karyotyping of fetal chromosomes
[28]	Evaluation of a multicentric collection of a 1-yr series of fetal samples with indication for prenatal invasive sampling simultaneously using three screening methodologies: (1) karyotype and QF-PCR; (2) two panels of MLPA; and (3) microarray-based analysis with a targeted BAC microarray. On a total of 900 samples, technical performance was excellent for karyotype, QF-PCR, and GMA (about 1% failure rate) but relatively poor for MLPA (10% failure). Mean turn-around time was 7 d for microarray or MLPA, 25 d for karyotype and 2 d for QF-PCR, with similar combined costs for each approach. A total of 57 clinically significant chromosomal aberrations were found (6.3%), with microarray yielding the highest detection rate (32% above other methods). The identification of VOUS (17, 1.9%) tripled that of karyotype and MLPA, but most alterations could be classified as likely benign after proving they were inherited
[29]	Evaluation of the results of prenatal microarray analysis on > 1000 fetal samples referred for testing and comparison of these data to published reports. Clinically significant CNVs were observed in 85/1115 cases (7.6%). Eighteen of the 1115 cases had VOUS (1.6%). Indications yielding the most clinically significant findings were abnormal karyotype/FISH (26/61, 42.6%), family history of chromosomal abnormality (13/137, 9.5%), abnormal ultrasound (38/410, 9.3%), abnormal serum screening (2/37, 5.4%) and advanced maternal age (5/394, 1.3%). Of 1075 cases having no previously known cytogenetic abnormality or family history, 18 (1.7%) had clinically significant genomic changes undetectable by conventional prenatal chromosome analysis
[30-33]	Papers reporting experience on a small number of samples and addressing the main issues in this field
[34]	Prospective cohort study of 243 women undergoing microarray testing alongside karyotyping when a structural abnormality was detected on prenatal ultrasound scan and review and meta-analysis of the literature. The collective number of samples analysed were 17113. The overall agreement between the two tests was 93.4% (95%CI: 90.4%-96.5%). The results obtained in attempting to calculate the rate of microarray detection over karyotyping were highly heterogeneous, ranging from 0.4% to 50%. When the indication was structural abnormality seen on ultrasound scan the detection rate over karyotyping was 10% (95%CI: 8%-13%). A sub analysis performed using cohorts published between 2011 and 2012 showed a lower detection rate (7%, 95%CI: 5%-10%). The authors suggest that GMA could have a higher detection rate not just in cases of abnormal scan findings but also with other indications for invasive testing, and conclude that it is likely that microarray testing will replace karyotyping in high risk pregnancies

BAC: Bacterial artificial chromosomes; CNAs: Copy number alterations; CGH-arrays: Comparative genomic hybridization-based arrays; CMA: Chromosomal microarray analysis; CNVs: Copy number variations; MLPA: Multiplex ligation-dependent probe amplification; PD: Prenatal diagnosis; QF-PCR: Quantitative fluorescent polymerase chain reaction; SNP-arrays: Single nucleotide polymorphism-based arrays; VOUS: Variants of uncertain (unknown) significance; ID: Intellectual disabilities.

interest in determining whether these technologies will offer similar advantages in the detection of fetal genomic imbalances in a prenatal setting.

DATA FROM THE LITERATURE

Starting in 2004, reports began to appear describing evi-

dence that array CGH could detect causative deletions and duplications in children with ID, and other disabilities and congenital malformations^[11] (Table 1).

Rickman *et al*^[12] have shown the feasibility of performing CGH-array for PD on DNA extracted from AF cells with the demonstration that in 29/30 samples, the CGH-array result was fully concordant with the karyotype.

Hillman *et al*^[13] published a critical appraisal of the literature evaluating the use of CGH-array in PD up to and including 2009. When CGH-array was used prenatally for any indication (*e.g.*, maternal age, parental anxiety or an identified ultrasound abnormality), pathogenic copy number variations (CNVs) or variants of uncertain (unknown) significance (VOUS) were detected in 3.6% (95%CI: 1.5%-8.5%) of cases in which conventional karyotyping was considered normal. When the indication for prenatal CGH-array was a fetal anomaly on ultrasound, microarrays detected an additional 5.2% (95%CI: 1.9%-13.9%) pathological CNVs or VOUS over conventional karyotyping. Heterogeneity (and hence large confidence intervals) was attributed to the varying resolution of the CGH-array methodology. In addition, there was considerable variation in the literature as to whether an attempt had been made to identify and investigate the presence of benign CNVs (by reviewing parental samples).

Other cohort studies, published from 2009 onwards, have demonstrated an increased detection rate over standard karyotyping ranging from 0.9% to 26.5%^[14-21]. Some of the studies with much larger detection rates may represent selection of patients rather than being a reflection of a true prospective series^[22].

More recently, Lee *et al*^[23] looked at the utility of both a 1-Mb BAC and 60-K oligonucleotide array in 3171 pregnancies. Although the added utility of CGH-arrays over karyotyping was small when there was an uneventful prenatal examination (0.52%), the proportion of cases in which additional information was provided by CGH-arrays increased to 8.2% when a fetus had an abnormality on ultrasound scan (USS).

Srebniak *et al*^[24] used a SNP-array with a resolution of 150/200 kb to analyze DNA from 207 cases with fetal anomalies, and detected additional information in 7.7% of cases, a similar percentage to that in the Lee and colleagues's cohort.

Over a period of 3 years (2008-2011), Wapner *et al*^[25] compared microarray with standard karyotyping in 4406 women undergoing PD for common indications, including advanced maternal age (46.6%), fetal abnormalities detected on ultrasonography (25.2%) and positive prenatal screening results (18.8%). Microarray analysis was performed using either a customized oligonucleotide-based microarray with spacing of approximately 1 probe per 75 kb, or a SNP-array with a comparable resolution. The analysis was successful in 4340 of 4391 cases with an adequate sample (98.8%), and identified all of the common autosomal and sex-chromosome aneuploidies and the unbalanced rearrangements detected by standard karyotyping in the 4282 non mosaic samples. As expected, microarray analysis did not identify balanced translocations (0.93% in this sample). The series also included seventeen triploid samples (0.4%), none of which were identified on microarray. Microdeletions or duplications of clinical significance were found in 96 of 3822 fetal samples with normal karyotypes (2.5%; 95%CI: 2.1%-3.1%), including 6.0% of cases in which fetal anomalies were detected on

ultrasonography. There were 94 copy-number variants of uncertain clinical significance that required adjudication by a Clinical Advisory Committee, and after discussion 61 (65%) were classified as pathogenic. A subsequent update of copy-number variants of uncertain significance resulted in reclassification of 30 copy-number variants as pathogenic and 8 as benign. With this additional information, the pathogenicity of 1.5% of copy-number variants detected on microarray analysis in karyotypically normal samples remained uncertain.

To assess whether chromosomal microarray analysis (CMA) improves the detection rate of prenatal chromosomal aberrations, Fiorentino *et al*^[26] explored the utility of microarray analysis in groups of pregnancies with *a priori* low risk for detection of submicroscopic chromosome abnormalities (usually not considered an indication for testing). A total of 3000 prenatal samples, including 2650 amniotic fluids (88.3%), 308 chorionic villus sampling (10.3%), 32 cultured amniocytes (1.1%), and 10 DNAs extracted by other laboratories from uncultured amniocytes (0.3%), were processed in parallel using both GMA and conventional karyotyping. The indications for prenatal testing included: advanced maternal age, maternal serum screening test abnormality, abnormal ultrasound findings, known abnormal fetal karyotype, parental anxiety, family history of a genetic condition and cell culture failure. Samples were processed using a whole-genome BAC platform with a resolution of about 1Mb across the genome and about 100 kb in 139 regions associated with constitutional disorders. In high risk groups (with abnormal ultrasound findings and fetal karyotype) the percentage of detection was 5.8% (7/120). In low risk groups the percentage was much lower: 0.5% (6/1118) in advanced maternal age and 0.7% (11/1674) in parental anxiety. No genetic imbalances were detected in any of the cases sampled for an abnormal maternal serum screening, nor for a family history of a genetic condition or chromosomal abnormality. A total of 24 (0.8%) fetal conditions would have remained undiagnosed if only a standard karyotype had been performed. About 17 (0.6%) of such findings would have otherwise been overlooked if CMA was offered only to high risk pregnancies.

Shaffer *et al*^[27] reported a study on 5003 prospective cases received from 2004 to 2011 for a variety of indications. The overall detection rate of clinically significant copy number alterations (CNAs) among unbiased, non-demise cases was 5.3%. Detection rates were 6.5% and 8.2% for cases referred with abnormal ultrasounds and fetal demise, respectively. The overall rate of findings with unclear clinical significance was 4.2% but would reduce to 0.39% if only *de novo* CNAs were considered. In cases with known chromosomal rearrangements in the fetus or parent, 41.1% showed CNAs related to the rearrangements, whereas 1.3% showed clinically significant CNAs unrelated to the karyotype. Finally, 71% of the clinically significant CNAs found by microarray were below the resolution of conventional karyotyping of fetal chromosomes.

In a comparative study of currently available methodologies for detection of chromosomal abnormalities after invasive prenatal sampling^[28], a multicentric collection of a 1-year series of fetal samples with indication for prenatal invasive sampling was simultaneously evaluated using three screening methodologies: (1) karyotype and QF-PCR; (2) two panels of MLPA; and (3) microarray-based analysis with a targeted BAC microarray. A total of 900 pregnant women provided informed consent to participate (94% acceptance rate). Technical performance was excellent for karyotype, QF-PCR, and GMA (about 1% failure rate) but relatively poor for MLPA (10% failure). Mean turn-around time was 7 d for microarray or MLPA, 25 d for karyotype and 2 d for QF-PCR, with similar combined costs for each approach. A total of 57 clinically significant chromosomal aberrations were found (6.3%), with microarray yielding the highest detection rate (32% above other methods). The identification of variants of uncertain clinical significance (17, 1.9%) tripled that of karyotype and MLPA, but most alterations could be classified as likely benign after proving they were inherited.

Breman *et al*^[29] evaluated the results of prenatal microarray analysis on > 1000 fetal samples referred for testing and compared these data to published reports. Clinically significant CNVs were observed in 85/1115 cases (7.6%) overall, and in 45/1075 cases (4.2%) if 40 abnormal cases with known chromosome abnormalities or familial genomic imbalances were excluded. Eighteen of the 1115 cases had variants of unclear clinical significance (1.6%). Indications yielding the most clinically significant findings were abnormal karyotype/FISH (26/61, 42.6%), family history of chromosomal abnormality (13/137, 9.5%), abnormal ultrasound (38/410, 9.3%), abnormal serum screening (2/37, 5.4%) and advanced maternal age (5/394, 1.3%). Of 1075 cases having no previously known cytogenetic abnormality or family history, 18 (1.7%) had clinically significant genomic changes undetectable by conventional prenatal chromosome analysis.

In 2013 several papers reported experience on a small number of samples and tried to address the main issues in this field^[30-33]. Finally, Hillman *et al*^[34] quite recently reported a prospective cohort study of 243 women undergoing microarray testing alongside karyotyping when a structural abnormality was detected on prenatal USS. This cohort is presented in the context of a systematic review and meta-analysis of the literature defining overall detection rates by microarray over karyotyping. When clinical indication for testing was abnormal fetal USS their cohort study noted a 4.1% increase in detection rate; lower than the rate of 10% (95%CI: 8%-13%) by meta-analysis. The VOUS rate was 2.1% (95%CI: 1.3%-3.3%) when the indication for GMA was abnormal scan. The VOUS rate was 1.4% (95%CI: 0.5%-3.7%) when any indication for prenatal GMA testing was meta-analysed. The authors, suggest that GMA could have a higher detection rate not just in cases of abnormal scan findings but also with other indications for invasive testing, and conclude that it

is likely that microarray testing will replace karyotyping in high risk pregnancies.

GUIDELINES

The accumulated evidence from many studies applying GMA together with chromosomal analysis in PD, demonstrate that there is improved detection of clinically significant genome imbalances when using GMA; proving the usefulness in using this technique in a PD setting. However, several issues remain to be addressed before implementing CGH-array in PD, such as: (1) in which pregnancies should CGH-array be carried out, whether for all pregnancies or only for pregnancies with ultrasound abnormalities; (2) which array platform to use; (3) an appropriate calling criteria must be established; (4) which confirmatory methods to use for the CGH-array findings; and (5) pretest counseling^[30].

Scientific societies have joined the discussions regarding microarray-based technologies in PD.

The American College of Obstetricians and Gynecologists stated that, although CGH-array has distinct advantages over classic cytogenetics in certain applications, the technology is not currently a replacement for classic cytogenetics in PD^[35].

The Genetics Committee of the Society of Obstetricians and Gynaecologists of Canada and the Prenatal Diagnosis Committee of the of the Canadian College of Medical Geneticists make three principal recommendations: (1) Array genomic hybridization is not recommended in pregnancies at low risk for a structural chromosomal abnormality; for example, advanced maternal age, positive maternal serum screen, previous trisomy, or the presence of "soft markers" on fetal ultrasound; (2) Array genomic hybridization may be an appropriate diagnostic test in cases with fetal structural abnormalities detected on ultrasound or fetal magnetic resonance imaging and could be done in lieu of a karyotype if rapid aneuploidy screening is negative and an appropriate turnaround time for results is assured; and (3) Any pregnant woman who qualifies for microarray genomic hybridization testing should be seen in consultation by a medical geneticist before testing so that the benefits, limitations, and possible outcomes of the analysis can be discussed in detail. The difficulties of interpreting some copy number variants should also be discussed. This will allow couples to make an informed decision about whether or not they wish to pursue such prenatal testing^[36].

The European Society of Human Genetics stated that arrays were of proven value for investigation of fetal abnormalities and encouraged the establishment of local guidelines for the use of genome-wide array analysis in the prenatal setting. The most important recommendations helpful when establishing local or national guidelines are: (1) Establish the indications for the use of genome-wide array analysis in the prenatal setting; (2) An array platform with a minimal resolution of 200 kb is recommended; (3) Laboratory specialists should have suf-

ficient experience with the interpretation of array results; (4) Parental blood sampling is highly recommended; (5) Pretest counseling, including providing written information and parental consent are a prerequisite; (6) The laboratory and the clinicians should agree on what to report and what not to report before offering array diagnostics; and (7) There should always be optimal communication between the laboratory specialists and the clinicians^[37].

The cytogenetics working group of the Italian Society of Human Genetics (SIGU) recommended the use of CMA in prenatal testing: (1) never as a substitute for conventional karyotyping; (2) for specific diagnostic purposes in selected pregnancies and not for general screening in all pregnancies; and (3) only in prenatal cases with specific indications, such as: (a) single (apparently isolated) or multiple sonographic fetal abnormalities; (b) *de novo* chromosomal rearrangements (even if apparently balanced) detected by standard karyotyping to investigate the possible presence of cryptic imbalance(s) related to the structural chromosome abnormality; and (c) supernumerary marker chromosomes in order to characterize their origin and genetic content^[38].

CONCLUSION

The amount of information that can be obtained from the human fetus is growing at a remarkable rate. Although the times when the fetus was regarded as a mysterious object are long gone, the concept that the fetus is a genetically distinct entity from the pregnant woman and that can be studied on an individual basis is fairly recent.

For both clinical and technical reasons, PD has always focused on chromosomal disorders, which represent a very important cause of prenatal morbidity and mortality^[39]. The standard cytogenetic techniques have been used for many years for the diagnosis of chromosomal defects, accompanied in recent years by molecular cytogenetic techniques. In the postnatal field, other techniques such as those based on microarrays have been proposed as a first level test in children with ID and MCA^[9].

Microarray-based techniques such as CGH-arrays and SNP-arrays allow detection of very small genomic imbalances (at the level of genes and even exons) that can determine pathological clinical conditions^[40-42]. In addition to these pathogenetic CNVs, there are other CNVs which represent normal variations, without negative effects on the phenotype. Moreover, many CNVs are associated with variable expressivity and incomplete penetrance, leading to a difficult prevision of the phenotype. Given the still incomplete knowledge of the so-called “variomat” (the set of all the changes in our genome) and the associated phenotypes, microarray-based testing is likely to identify variants of uncertain and unknown clinical significance. The interpretation of these variants is a challenge for medical geneticists, who often find it difficult to establish precise correlations between genotype and phenotype.

These difficulties, already significant in a postnatal context, become critical in the prenatal setting, where the

fetal phenotype is difficult to explore and where there are huge dilemmas regarding the advice to be given. For these reasons, despite increasing interest in applying these techniques in PD, their actual use is as yet not widespread. The use of microarray-based techniques in PD is currently a topic of much debate, between supporters of the technology and its application and those that recommend a more cautious approach.

One of the most important issues concerns the pregnancies to be considered for this test. Is it appropriate and convenient to apply the routine examination by microarray in all pregnancies that are subjected to invasive PD or it is better to restrict their use to pregnancies that have particular characteristics, such as the presence of fetal ultrasound abnormalities? The currently available data does not support the implementation of these methods in low-risk pregnancies; however, under other conditions they are clearly advisable, as indicated in the Position Statement of the cytogenetic working group of the SIGU^[38].

Moreover, the number of chromosome abnormalities not detectable by microarray analysis suggests that microarray technology should remain a complementary analysis and not a replacement for current PD tests^[43].

Regarding the choice of platform, there have been no systematic studies to identify a specific platform most suitable for PD. The difficulty lies in finding the appropriate resolution, which must be high enough to detect small imbalances (already identified as a possible cause of disease patterns) but not so high as to generate large numbers of CNVs of uncertain significance. To address this problem, some groups have opted for the use of targeted platforms, which show only well-characterized imbalances linked to specific clinical situations. Although this approach avoids many of the problems presented to the examiner, it severely limits the diagnostic power of the technique (one of the main arguments for its introduction). In addition, the knowledge of CNVs is rapidly expanding and ever new microdeletion/microduplication syndromes are being discovered and described. This would involve a continuous update of the targeted platforms, which is practically unfeasible. A fair compromise could be represented by the platforms with an acceptably high (but not overly high) resolution of the entire genome (at least 200 kb) with a greater number of probes in regions of particular clinical interest^[37,44].

While oligonucleotide arrays with high-density exonic coverage remain the gold standard for the detection of CNVs, SNP-arrays allow for detection of uniparental disomy and consanguinity, while also providing a higher sensitivity in detection of low-level mosaic aneuploidies^[11]. Moreover, SNP-arrays allow identification of polyploidies and chimerisms.

There is increasing interest in the use of mixed platforms (oligo-SNP), which combine the advantages of the oligonucleotides in terms of diagnostic accuracy of CNVs, with those of the SNPs. These platforms deserve a thorough evaluation on a large number of cases and may become the best choice for PD.

Another important point to consider is the information process. PD is an extremely delicate issue, and any defect in communication between doctor and patient can produce very serious problems. In particular, genetic counseling relating to the examination by microarray in a prenatal environment is difficult and represents a challenge for even the most experienced geneticist. Therefore, there is a clear need for specific training and draft guidelines that will help to improve and standardize the professional standards in this sensitive area.

In conclusion, genomic rearrangements represent an important aspect of human pathology and the application of microarray-based techniques for diagnosis is likely to continue growing in significance. Given the undeniable advantages of these techniques over conventional cytogenetics, there is an increasing pressure towards their application in PD. However, introduction of these technologies into clinical practice should proceed with caution and be offered only by experienced laboratories and after proper validation, showing robust, reliable and reproducible results^[26]. While there is sufficient evidence in the literature to recommend the use of these technologies in specific conditions, it is important to avoid an unchecked drift towards widespread use driven by commercial interests. It is critical that application is tightly regulated and that scientific societies remain vigilant and participate in the decision making process.

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P- Reviewer: Chen JJ S- Editor: Zhai HH
L- Editor: A E- Editor: Zheng XM



Genetic alterations in head and neck squamous cell carcinoma: The next-gen sequencing era

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Received: June 28, 2013 Revised: October 28, 2013

Accepted: November 7, 2013

Published online: November 27, 2013

Abstract

Head and neck squamous cell carcinoma is the sixth most common cancer in the world with approximately 650000 new cases diagnosed annually. Next-generation molecular techniques and results from phase 2 of the Cancer Genome Atlas becoming available have drastically improved our current knowledge on the genetics basis of head and neck squamous cell carcinoma. New insights and new perspectives on the mutational landscape implicated in head and neck squamous cell carcinoma provide improved tools for prognostication. More importantly, depend on the patient's tumor subtypes and prognosis, deescalated or more aggressive therapy maybe chosen to achieve greater potency while minimizing the toxicity of therapy. This paper aims to review our current knowledge on the genetic mutations and altered molecular pathways in head and neck squamous cell carcinoma. Some of the most common mutations in head and neck squamous cell carcinoma reported by the cancer genome atlas including TP53, NOTCH1, Rb, CDKN2A, Ras, PIK3CA and EGFR are described here. Additionally, the emerging role of

epigenetics and the role of human papilloma virus in head and neck squamous cell carcinoma are also discussed in this review. The molecular pathways, clinical applications, actionable molecular targets and potential therapeutic strategies are highlighted and discussed in details.

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Key words: Head and neck squamous cell carcinoma; TP53; Ras/PIK3CA; NOTCH1/p63; HPV/E6/E7/Rb/CDKN2A

Core tip: Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world with approximately 650000 new cases diagnosed annually. Understanding the molecular pathways that are implicated in the pathogenesis of HNSCC enable clinicians to be able to classify and to prognosticate the disease based on subtypes, such as human papilloma virus (HPV)-positive vs HPV-negative HNSCC. More importantly, patients may be placed on de escalated or more aggressive therapies depend on their tumor subtypes and prognosis. This paper aims to review our current knowledge of the most common genetic alterations in HNSCC.

Nguyen TK, Iyer NG. Genetic alterations in head and neck squamous cell carcinoma: The next-gen sequencing era. *World J Med Genet* 2013; 3(4): 22-33 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i4/22.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i4.22>

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world with approximately 650000 new cases diagnosed annually^[1]. Lesions in

the head and neck area impair both forms and functions significantly. Surgery, one of the three pillars of treatments, is not only technically demanding but surgery also presents difficult challenges during rehabilitation in the postoperative period. On the other hand, radiotherapy is associated with significant complications and side effects that render the treatments intolerable for many patients. Thus, understanding the molecular pathways that are implicated in the pathogenesis of HNSCC enables clinicians to be able to classify the disease based on subtypes, such as human papilloma virus (HPV)-positive vs HPV-negative HNSCC and to prognosticate better. More importantly, patients may be placed on deescalated or more aggressive therapies depend on their tumor subtypes and prognosis. Moreover, chemotherapy has been useful to prevent recurrence in other cancers such as breast and may become relevant in HNSCC when an actionable target is discovered. The aim of this paper is to review our current knowledge of the molecular pathogenesis of HNSCC-knowledge made available by advanced, next-generation molecular techniques. We also discuss how this growing body of evidence currently shapes research interests and research directions in the quest of finding better treatments for HNSCC.

CELLULAR PROLIFERATION

TP53

TP53 encodes for the p53 protein and is widely touted as “the guardian of the genome” due to its master regulatory role in monitoring DNA damage, promoting senescence, inducing cell cycle arrest and apoptosis. Early studies revealed that somatic mutations in TP53 are found in 47% to 70% of HNSCC making TP53 mutations the most commonly mutated genes implicated in HNSCC^[2-5]. Smoking and alcohol, two well-known causes of TP53 mutations are the leading risk factors in HNSCC^[6,7]. Furthermore, evidence suggests that these mutations occur relatively early in the course of HNSCC development. Premalignant dysplastic lesions for HNSCC such as leukoplakia contain TP53 mutations in as high as 27% of cases^[8]. Additionally, the presence of p53 mutations in these premalignant lesions also increases the risk of progression to invasive carcinoma^[8,9]. It is important to point out that such precursor lesions indicate a field defects and that both clonal and non-clonal TP53 mutations can be found in macroscopically normal epithelium^[5]. In as high as 35% of oral and oropharyngeal carcinomas, the primary tumor is surrounded by mucosal epithelium that contains TP53 mutations^[10].

TP53 is not only a significant determining factor in the carcinogenesis but patients with TP53 mutations also have worse prognoses. In a study by Poeta *et al*^[11] HNSCC patients with disruptive TP53 mutations have a decreased survival rate of more than 1.5 fold when compared with TP53 wild-type HNSCC. More specifically, a truncating TP53 mutation is associated with a worse overall survival and progression-free survival^[12].

These could be due to several factors. Firstly, disruptive mutations of TP53 can lead to a complete shutdown of intracellular restorative processes. TP53 mutants also disrupt tissue architecture^[13], upregulate angiogenesis^[14], as well as participate in migration, invasion and metastasis^[15,16]. All these factors contribute to a much more aggressive tumor biology. Secondly, TP53 mutants are extremely resistant to treatments. Several prospective trials have shown that patients with TP53 mutations respond poorly to cisplatin and fluorouracil^[17,18]. Additionally, field defects with TP53 mutations are found to often present in surgical margins during tumor resection^[19]. Retrospective studies have shown that both local recurrence and metachronous primary can arise from within the field cancerization^[20,21]. Thus, because of TP53 mutants within the field cancerization, an R0 surgical margin may not result in improved survival. Both primary and adjuvant radiotherapy are found to be less effective in HNSCC patients with TP53 mutations and have a much higher rate of locoregional recurrence and failure rate, respectively^[22,23].

Given the significant role of TP53 mutations in HNSCC and associated clinical implications discussed above, considerable efforts have been devoted to explore treatment strategies. This has proven to be challenging due to the wide spectrums of mutation patterns in TP53. Missense mutations in the DNA binding domain of the p53 protein are the most common type of TP53 mutations and account for 50%-70% of mutations^[2,3,24]. Other patterns of TP53 mutations have also been described, for example, 16% nonsense, 16% insertion or deletion and 8% splice site mutation in one series^[1]. Although most of these mutations are loss-of-function mutations, gain-of-function oncogenic activities associated with TP53 mutations have also been documented. In fact, for almost an entire decade after it was first discovered, TP53 was considered a proto-oncogene^[25]. These gain-of-function mechanisms remain poorly understood. Loyo *et al*^[26] suggest that such gain-of-function activities arise from the interactions of defect p53 with other regulatory proteins. Examples of p53 gain-of-function activities are the interactions of p53 with p63/p73^[25], ability of TP53 mutants to escape growth arrest induced by v-Ki-ras2 Kirsten rat sarcoma viral oncogene^[27], and the ability to promote invasion and metastasis *via* integrin and EGFR upregulations^[15].

Thus, given such complex and paradoxical activities of p53 mutants, several therapeutic strategies have been proposed and tested in clinical trials. One strategy aims to restore wild-type p53 functions in tumor cell. This stems from an important proof-of-principle in several mouse models in which reactivating wild-type p53 functions results in tumor regression^[28]. For example, adenovirus gene therapy can reactivate wild-type p53 functions. Clinical trials studying adenoviral based treatment such as Advexin (Introgen Therapeutics Inc., Austin, TX) and ONYX-015 (Onyx Pharmaceuticals Inc., San Francisco, CA) have yielded some positive results in phase I, II and several pending results in phase III^[29,30]. In a trial that assessed

response to the treatment of ONYX-015 in combination with cisplatin and 5-fluorouracil, the complete and partial response rates were 27% and 36% respectively. Patients with ONYX-015 injection also have longer time to tumor progression^[30]. This trial also yielded some other important findings: (1) patients with documented chemoresistance have objective tumor regression after injection with ONYX-015; and (2) side effects include flu-like symptoms, injection site pain and mucous membrane disease. ONYX-015 has recently been approved for treatment of HNSCC in China^[31]. Furthermore, there are other innovative approaches to reactivate wild-type p53. These approaches revolve around our progressively expanding knowledge of the biomechanism within the p53 pathway: by targeting the MDMX-p53 ubiquitination pathway^[28], by using proteasome inhibitor bortezomib^[32], or by using p53 reactivating molecules such as PRIMA-1^[33,34]. Another avenue of approach for therapy in HNSCC patients with TP53 mutations is to target p53 mutants directly. As discussed above, high levels of mutant p53 is critical in tumorigenesis. Thus, molecules that can destabilize or reduce mutant p53 turnover may be beneficial for HNSCC patients with TP53 mutations. Li *et al.*^[35,36] have shown that p53 mutants level can be reduced by inhibiting either Hsp90 or HDAC6. This approach maybe quickly translated into clinical use with the availability of Vorinostat (Merck & Co., White House Station, New Jersey), an FDA-approved HDAC6 inhibitor that is widely used for lymphoma and other solid tumors^[35].

Rb/CDKN2A (p16)

The Retinoblastoma (Rb) protein, located on chromosome 13, is a critical regulatory protein of the G1 checkpoint. The Rb pathway is described schematically in Figure 1. In healthy cells, hypophosphorylated Rb protein forms a complex with the transcription factor E2F to promote G1 arrest. Cells progress to S-phase after hyperphosphorylation of Rb occurs. Thus, epithelial carcinogenesis in HNSCC is thought to arise from excessive hyperphosphorylation of Rb proteins. This event can occur with mutations in several different loci. Rb mutations can happen due to loss of heterozygosity (LOH) or microsatellite instability (MI)^[37]. LOH has been implicated in many malignancies, including HNSCC^[38,39]. The rate of LOH in Rb gene is as high as 60% in laryngeal SCC in some series whereas MI is reported at 34% in a series of stage 2 laryngeal SCC patients^[39]. Although the inactivation of Rb gene product (pRb) due to viral oncogenes in HPV-positive HNSCC is well established, the inactivation of pRb as a result of LOH/MI in HPV-negative HNSCC is much more contentious. Recent evidence suggests that a mutation at one Rb locus is not enough to stop Rb expression and that loss of pRb expression only arises when there are mutations of both Rb alleles^[37]. This study also suggests that despite the high frequency of LOH or MI in this series, such presence does not offer additional information to tumor biology or patient prognosis^[37]. Rather, a multi-step

tumorigenesis in which Rb is an intermediary is a more likely process *in vivo*.

Cyclin-dependent kinase inhibitor 2A (CDKN2A) at 9p21 locus controls the phosphorylation of Rb. In the presence of CDKN2A, cyclin-dependent kinases CDK4 and CDK6 are prevented from phosphorylating Rb. Without this regulation, Rb-E2F complexes become destabilized due to hyperphosphorylation and progression to S phase proceeds unchecked^[40]. The Cancer Genome Atlas (TCGA) reported a mutation rate of 21% in the CDKN2A locus in HNSCC^[4]. LOH at the CDKN2A locus is common in premalignant oral lesions such as leukoplakia and can be found in as high as 80% of HNSCC tumors^[41-43]. CDKN2A encodes for two tumor suppressors: p16 and p14. In HPV-negative hypopharyngeal and oropharyngeal SCC, p16 downregulation and concurrent cyclin D1 overexpression have been linked with poorer outcome^[44,45]. Furthermore, an alternate reading frame p14 has been shown to be associated with a slightly higher risk of developing a second primary malignancy after an index HNSCC^[46]. Such recent evidence, together with the feasibility of detecting p14 and p16, have led to increased interest in detecting p14 and p16 as surrogate markers and as prognostication tool in HNSCC^[45,46].

Evidence of tumor arrest after transfection of p16 in negative p16 squamous carcinoma cell lines has offered some therapeutic directions^[47,48]. The demethylating agent 5-aza-2'-deoxycytidine has been shown to recover p16 expression^[49]. Moreover, 5-aza has also been shown to increase the radiosensitivity of HNSCC tumors^[50].

TERMINAL DIFFERENTIATION

NOTCH1/p63

The discovery of NOTCH1 as a commonly mutated gene in HNSCC owes much to the availability of next-generation sequencing. NOTCH 1, a large gene of 34 exons, was first shown to be involved in tumorigenesis while studying T-cell leukemias^[51]. Further evidence emerged and different patterns of NOTCH1 mutations have been found to be associated with lung cancer, various forms of leukemia and HNSCC^[2,52,53]. TCGA reports a mutation rate of 19% for NOTCH1 in HNSCC^[4]. Function of NOTCH1 is highly contextual in normal biology as well as in pathology. In normal biology, activation of NOTCH1 causes terminal differentiation in some tissues while performs stem cell maintenance in other tissues^[54]. From studies of T-cell lymphoblastic and chronic lymphocytic leukemia, the NOTCH1 pathway is found to be upregulated and thus becomes oncogenic. However, reduced NOTCH1 signaling has been found in HNSCC, suggesting tumor suppressing activities of NOTCH1 in these cell lines^[2,3]. Several animal models have also been shown to support this paradoxical biological duality of NOTCH1 in tumorigenesis^[55-57]. Unsurprisingly, NOTCH1 mutations in HNSCC are fundamentally distinctive from oncogenic mutations found in other types of cancers. The majority of NOTCH1 mutations in HNSCC were found in N-ter-

HNSCC treatment. Interestingly, it led to a paradoxical discovery that EGFRvIII was a surprised biomarker of improved disease control with a caveat that the sample size was small^[81]. Successful phase I trials for ABT-806, a next generation of EGFR mAbs, have paved way for some recent phase II trials with pending results^[82-84].

Understanding the mechanisms of EGFR resistance in both treatment-naïve and treatment-experienced settings holds the key to unlock many translational opportunities. A phenomenon observed to be highly correlated with cetuximab resistance and worse disease progression is the epithelial-to-mesenchymal transition (EMT)^[85]. Interestingly, early evidence suggests that reversing EMT will re-sensitize resistant HNSCC cells to cetuximab and TKIs such as gefitinib^[86,87]. Moreover, agents that specifically kill EMT transformed cells such as salinomycin, may be synergistic with cetuximab^[88]. Thus, the EGFR pathway remains to be a very promising domain to investigate for the next generation targeted treatment for HNSCC.

Ras/PIK3CA

Conflicting early evidence reported a rate of mutations of HRAS gene in 35% of oral cancers from in India but none in the United States^[89,90]. Recently, with the availability of deep sequencing technique, HRAS mutations have been shown to be among the most common mutations in HNSCC in the United States with the incidence of 3% to 5%^[2-4]. HRAS is the only one of the three Ras genes found to be implicated in HNSCC^[91]. These three isoforms of Ras proteins exhibit tissue-specific functions due to differences in the C-termini that determine their lineage-specific roles^[92]. Because attempts to directly inhibit the Ras signaling in clinical trials have been disappointing, this review will focus on newer research direction in investigating a major downstream effectors of Ras: the Phosphoinositide-3 kinase (PI3K) pathways^[93,94]. As shown in Figure 2, the PI3K pathway is downstream of Ras and is important for cell growth and survival^[95].

The PI3K pathway can become over activated by PIK3CA mutations or as shown in Figure 2, by a loss of inhibition from phosphatase and tensin homolog (PTEN), a negative regulator. Two “hot spot” domains in the PIK3CA gene contain activating mutations in 6%-11% of HNSCC^[96,97]. On the other hand, LOH of PTEN is as frequent as 40% of HNSCC^[98]. Evidence suggests that LOH alone is adequate to drive tumorigenesis^[99]. LOH mechanisms can arise from either epigenetic or silencing somatic mutations^[100]. Regardless of the how, when the PI3K pathway becomes over activated, many broad downstream effects occur: angiogenesis, increased metabolism, enhanced proliferation and apoptosis inhibition. Akt and its downstream agent mTOR have been implicated in these downstream effects^[101-103].

Targeting the PI3K/AKT/mTOR axis has been shown to show some positive responses of tumors to treatments^[104]. This pathway can be targeted at multiple different targets for therapy. Currently, a pilot trial investigating the

neoadjuvant use of Rapamycin, a known mTOR inhibitor, to treat advanced HNSCC patients is being conducted^[105]. Other mTOR inhibitors such as everolimus or temsirolimus are also available for testing. On the other hand, MK2206, an Akt inhibitor, is also being tested in recurrent or metastatic HNSCC patients^[106]. Existing PI3K inhibitors such as PX-866 or BKM120 are also being tested alone, or in combination with paclitaxel, docetaxel or cetuximab in recurrent and metastatic HNSCC^[107-111]. The basis for trials that investigate PI3K inhibitors together with cetuximab stems from an observation that PI3K amplification may have causes resistance to EGFR inhibition^[101].

EPIGENETICS

Deep exome sequencing studies of HNSCC cell lines have found, at considerable frequencies, mutations in several genes that act at an epigenetic level. These genes are MLL2, NSD1 and SYNE1^[2,3]. According to TCGA, the rates of mutation for MLL2 and NSD1 in HNSCC are 18% and 11% respectively^[4]. Both MLL2 and NSD1 code for histone methyltransferases. Studies that specifically investigate MLL2 and NSD1 in HNSCC are currently lacking. However, MLL2 have been reported to be a major tumor suppressor gene in non-Hodgkin lymphoma and inactivating somatic mutation of MLL2 is indicated as a driver mutation for this malignancy^[112]. Other histone modifications enzymes have also been found to be associated with solid human cancers such as renal cell carcinoma (RCC), breast, gastric and colorectal carcinomas^[113-115]. Histones acetyltransferases p300/CBP mutations are found in solid and hematological tumors, also suggesting their involvements in critical tumorigenic pathways^[116]. A study by Yang *et al*^[117] from China reported histone modification as a major step in the pathogenesis of laryngeal carcinoma. Histone modification enzymes in HNSCC remain to be a new frontier for research.

Besides histone modifications, DNA methylation is another form of epigenetic regulation. DNA methylation blocks transcription factors from binding to initiate transcription complex formation. Furthermore, methylated DNA sequences also have a higher affinity for histone modification enzymes and recruitments of these enzymes induce genes silencing^[118,119]. Body of evidence that links abnormal DNA methylation to HNSCC is well established. Many genes that are involved in cell cycle, cell-cell adhesion, migration, angiogenesis and metastasis in HNSCC cell lines have been found to be associated with DNA methylation. Examples of these genes are p15, p16, cyclin A1, RAR-B2, CDKN2A, E-cadherin, DAPK and others^[120-124]. Interestingly, DNA hypermethylation is only one side of the story: DNA hypomethylation has also been implicated in laryngeal carcinoma from investigating S100A4. The gene S100A4 has been reported as an important mediator of EMT and metastasis^[125-127]. Recent evidence has suggested that S100A4 is also important in the maintenance and development of

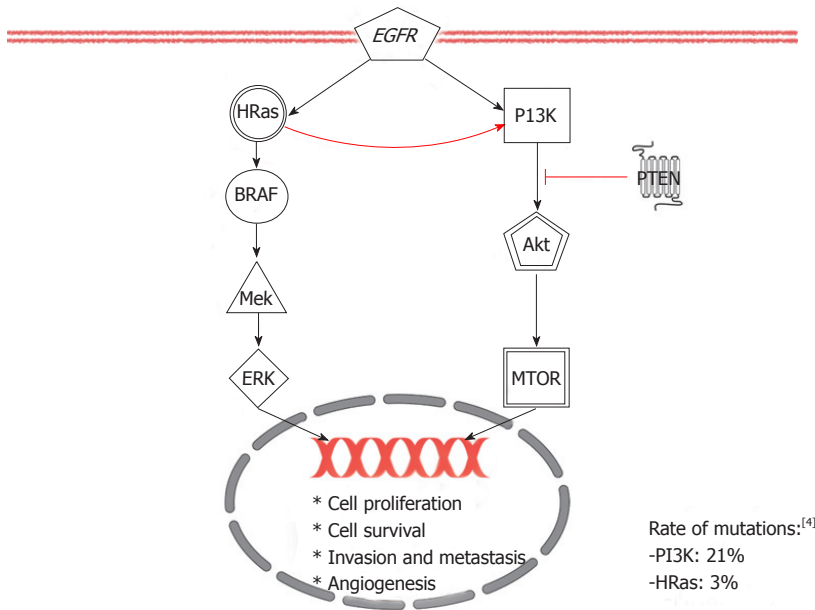


Figure 2 The Ras/PI3K pathway. EGFR: Epidermal growth factor receptor; Mek: Methyl ethyl ketone; MTOR: Mammalian target of rapamycin.

head and neck cancer-initiating cells (CIC). In this CICs population, S100A4 promoter is hypomethylated^[127]. Other supporting evidence for hypomethylation of S100A4 exists as well: demethylating agents induce both S100A4 RNAs and proteins expressions^[128]. A possible explanation for this methylation paradox is that when DNA methylation becomes aberrant and an imbalance occurs, global hypomethylation could lead to activation of oncogenes whereas focal hypermethylation can silence tumor suppressor gene.

In the genetic landscape of HNSCC, activating of oncogenes is an exception rather than the norm. In principle, the majority of HNSCC harbored inactivating mutations in tumor suppressor genes. Results of HNSCC treated with agents that modulate epigenetic regulations such as histone deacetylase (HDAC) inhibitors and demethylation agents are encouraging and lend support to this observation. Demethylation treatments have been shown to restore tumor suppressor gene functions, arrest tumor growths, and increase radiosensitivity of HNSCC cells^[49,50,129]. Additionally, HDAC inhibitors have also yielded some promising results. Valproic acid (VPA), a relatively weak HDAC inhibitors, have been shown to inhibit both acute and chronic growth of HNSCC cells^[130]. VPA has also been shown to improve tumor arrest when used together with a recombinant adenovirus in an HNSCC xenograft mouse model^[131]. A phase 2 trial currently evaluates the addition of VPA to standard platinum-based chemoradiation^[132]. Another HDAC inhibitor, Vorinostat, is currently in phase 1 for stage III and IV SCC of the oropharynx and in phase 2 for combination with capecitabine in recurrent and metastatic HNSCC^[133,134].

HPV

Both from a genetic and a clinical perspective, HPV-

positive HNSCC is a distinct entity from HPV-negative HNSCC. Most importantly, the overall prognosis of HPV-positive HNSCC is much more favorable than HPV-negative HNSCC^[135,136]. This presents an opportunity to carry out de escalated therapies to minimize treatment related toxicities with ongoing trials investigating this strategy^[137]. The pathogenesis of HPV is due to viral oncoproteins E6 and E7 inactivating tumor suppressors p53 and Rb. E6 targets p53 and E7 targets Rb, as shown in Figure 1 and cause ubiquitin-dependent protein degradation^[138]. Understanding the mechanism of E6 and E7 has led to some important applications. Because E7 degrades Rb, it also leads to an upregulation of p16, an upstream regulator of Rb. Thus, p16 has been used as a biomarker to diagnose HPV-positive HNSCC^[138,139]. Moreover, E6 and E7 are appealing molecular targets for therapy. In HPV-positive HNSCC cells lines, short hairpin RNAs that target and suppress E6 and E7 have been shown to restore the level of p53 and Rb^[140]. Researches investigating how E6 and E7 can be inhibited *in vitro* are at early stages. Two strategies currently exist: disruption of E6/E7 binding with its ubiquitin ligase enzyme or blocking the activation of downstream ubiquitin/proteasome systems (UPS)^[141,142]. So far, two trials have shown that Bortezomib, an UPS inhibitor, has a very poor response rate in locally recurrent or advanced HNSCC. However, it must be pointed out that the rate of HPV-positive was low in one trial (1 out of 20 tumors) and was not reported in the other trial^[143,144]. Thus, further studies and trials are indeed necessary in this area. Immunotherapy for HPV-positive patients with HNSCC is another area of active research. Proof of principle studies, mainly in mouse models, have demonstrated that engaging CD8⁺ T cell response to target E6/E7-specific antigens have led to tumor eradication^[145,146]. In HPV-16-positive oropharyngeal cancers, improved adaptive immunity as measured by CD8 cell counts is associated

with a better prognosis^[147]. Besides immunotherapy, vaccination is also gaining traction when HPV vaccines are now being recommended by the Center for Disease Control and Prevention for the prevention of anogenital and oropharyngeal cancers in male. Thus, the role of immunotherapy is becoming more and more relevant in HPV-positive HNSCC.

CONCLUSION

Next-gen sequencing has allowed us to accumulate an unprecedented amount of knowledge about mutations found in HNSCC. In summary, we can make the following observations. Firstly, inactivation of tumor suppressor genes is much more common in HNSCC cells than activation of oncogenes. Secondly, it is unlikely that a single target therapeutic approach will work and patients will benefit more from agents that can target more than one receptor or from combination therapy. Thirdly, mutations in HNSCC are heterogenous with complex interplay between many different molecular pathways at both the genetic and epigenetic levels. In our humble opinion, this heterogeneity should be seen as opportunity rather than obstacle. It seems inevitable that as our knowledge continues to expand and becomes more refined, we will be able to classify HNSCC into subtypes based on the pattern of mutations. By classifying into subtypes, we will be able to improve our ability to diagnosis, stage, and prognosticate. More importantly, we will be able to give therapy with greater potency and less toxicity. Certainly, this is already happening to an extent with HPV-positive and HPV-negative HNSCC. As we identify more biomarkers and invent new therapies to target these biomarkers, the trend in management of HNSCC continues its shift towards a more personalized therapeutic approach.

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P- Reviewer: Mandic R S- Editor: Song XX
L- Editor: A E- Editor: Liu XM



Regulation of the cell fate by DNA damage and hypoxia

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Author contributions: All authors contributed substantially to conception and design, acquisition of data described in the articles, drafting the article, designing the figures of the manuscript and revising it critically for important intellectual content, and approved the final version to be published.

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Received: June 13, 2013 Revised: September 6, 2013

Accepted: September 18, 2013

Published online: November 27, 2013

Abstract

In order to provide the means for the design of novel rational anti-cancer drug therapies research efforts are concentrated on unravelling the molecular circuits which induce programmed cell death and block proliferation of cancer cells. Modern therapeutic strategies are based on the understanding of the complexity of physiological functions such as differentiation, development, immune responses, cell-cycle arrest, DNA damage repair, apoptosis, autophagy, energy metabolism, and senescence. It has become evident that this knowledge will provide the means to target the components of the pathways involved in these processes in a specific and selective manner thus paving the way for the development of effective and personalised anti-cancer therapies. Transcription is a crucial cellular process that regulates a multitude of physiological functions, which are essential in disease

progression and cellular response to therapy. Transcription factors such as the p53 tumor suppressor and the hypoxia-inducible factor- α (HIF- α) are key players in carcinogenesis and cellular response to cancer therapies. Both of these transcription factors regulate gene expression of genes involved in cell death and proliferation, in some cases cooperating towards producing the same outcome and in some others mediating opposing effects. It is thus apparent that fine tuning of the activity of these transcription factors is essential to determine the cellular response to therapeutic regimens, in other words whether tumor cells will commit to apoptosis or evade engagement with the anti-proliferative effects of drugs leading to drug resistance. Our observations support the notion that the functional crosstalk between HIF-1 α and p53 pathways and thus the fine tuning of their transcriptional activity is mediated by cofactors shared between the two transcription factors such as components of the p300 co-activator multiprotein complex. In particular, there is evidence to suggest that differential composition of the co-modulatory protein complexes associated with p53 and HIF-1 α under diverse types of stress conditions differentially regulate the expression of distinct subsets of p53 and HIF-1 α target genes involved in processes such as cell cycle arrest, apoptosis, chronic inflammation, and cellular energy metabolism thereby determining the cellular fate under particular types of micro-environmental stress.

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Key words: Cancer; Transcription; Apoptosis; Inflammation; Tumor energy metabolism; Glycolysis; Oxidative phosphorylation; p53; Hypoxia-inducible factor; p300/CBP associated factors

Core tip: The results of our work endorse the notion that specific features determine targeting of transcription factors to distinct clusters of their target genes including the nature of the DNA binding sites found within the regulatory region of the promoter of each one of the target genes, the composition of the cofactor

network associated with different transcription factors under diverse types of stress conditions and the precise posttranslational modifications of each one of the transcription factors linking characteristic PTM codes with discrete types of micro-environmental stress. These features are essential considerations for the design of effective therapeutics and individualised cancer treatment.

Rajendran R, Krstic-Demonacos M, Demonacos C. Regulation of the cell fate by DNA damage and hypoxia. *World J Med Genet* 2013; 3(4): 34-40 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i4/34.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i4.34>

INTRODUCTION

The transcriptional regulation of gene expression is a crucial mechanism by which cells maintain homeostasis, differentiate, survive and proliferate, respond to internal signals as well as those they receive from their surroundings, and adjust to local environmental conditions^[1]. The transcription process is regulated mainly at two levels. One encompassing transcription factors and the transcriptional machinery, and the other involving chromatin which is the packaging structure of the DNA and consists of the four histone proteins H2A, H2B, H3 and H4 forming the nucleosome^[2,3]. The two levels of regulation are connected to each other since access of the transcription machinery to the DNA is regulated by molecular modifications of the chromatin structure executed by remodelling reactions such as phosphorylation, methylation, and acetylation^[4] which control the binding between transcription factors and DNA thereby selectively and specifically modulating gene expression of their target genes^[5,6]. These modifications represent the so called "histone code"^[7], which is a type of encryption that indicates either open access (euchromatin structure) of transcription factors to the DNA and transcription initiation of the target genes or closed chromatin conformation (heterochromatin) and transcriptional repression^[8,9]. In this respect transcriptional co-factors, which are proteins mediating histone modifications thus determining the open or closed chromatin conformation are of crucial importance in the activation or repression of gene expression and therefore for the cellular physiology^[10-12].

The detailed understanding of the regulation of gene expression has provided the means to comprehend how aberrant regulation of the transcriptional events can lead to disease^[13]. The role of DNA binding transcription factors and their modulators, of the non-coding RNAs, as well as the effects of epigenetic changes on the structure of the chromatin on transcription regulation and the impact of these events on the cellular physiology has been elucidated for many different diseases, for example diabetes^[14] cardiovascular disease^[15], neurological disorders^[16], rheumatoid arthritis^[17] cancer^[18] and conditions

such as obesity^[19] and ageing^[20]. Transcriptional regulation is not only important to understand the initiation, development, and prognosis of the disease but it is also imperative in predicting the cellular response to therapeutic modalities^[21-24].

DNA DAMAGE RESPONSE: THE ROLE OF THE p53 TUMOR SUPPRESSOR

A characteristic example of the importance of the transcription process in the outcome of the disease and the cellular response to drug treatment has been demonstrated by the function of the transcription factor and tumor suppressor protein p53^[25]. p53 is a transcription factor responding alternatively to diverse types of stress conveying different signals in a manner dependent on the type of stress^[26] by modulating gene expression of specific subsets of its target genes involved in vital and sometimes contradicting cellular functions such as cell cycle control^[27], apoptosis^[28], senescence^[29,30], autophagy^[31], DNA damage repair^[32,33], and tumor energy metabolism^[34]. It is worth noting that more than 90% of p53 mutations in human cancers occur in its DNA binding domain^[35] hampering the ability of this transcription factor to bind to DNA and transactivate its transcription target genes and emphasising the importance of transcription in oncogenesis^[36]. Under mild stress conditions p53 facilitates cell survival by activating a set of genes involved in cell cycle arrest and DNA damage repair^[37]. In prolonged stress or irreversible DNA damage p53 activates programmed cell death^[38]. Post-translational modifications of p53 including ubiquitination, phosphorylation, methylation and acetylation are also very important in the regulation of its protein stability and transcription target selectivity^[39].

HYPOXIA-INDUCIBLE FACTOR-1 α MEDIATED RESPONSE TO HYPOXIA

Hypoxia is an important pathophysiological state found mainly in solid tumors since the rapid growth of cancer tissues is associated with vascularisation deficiency, and therefore low oxygen availability which reaches levels below 5%^[40]. Hypoxic conditions give rise to the expression of genes encoding proteins which promote angiogenesis, invasion and metastasis, and enhanced glycolytic metabolism^[41-45]. Major contributing factors to the cellular and systemic adaptation in response to hypoxic conditions are primarily the hypoxia-inducible factors (HIFs)^[45,46]. HIF-1 is a transcription factor that regulates the induction of various genes facilitating adaptation and survival of cells in low oxygen conditions such as erythropoietin^[47] vascular endothelial growth factor^[48] glucose transporters, and glycolytic enzymes^[49,50].

CROSSTALK BETWEEN p53 AND HIF-1

The functional crosstalk between HIF-1 α and p53 pathways at several levels has been extensively studied^[51-53]

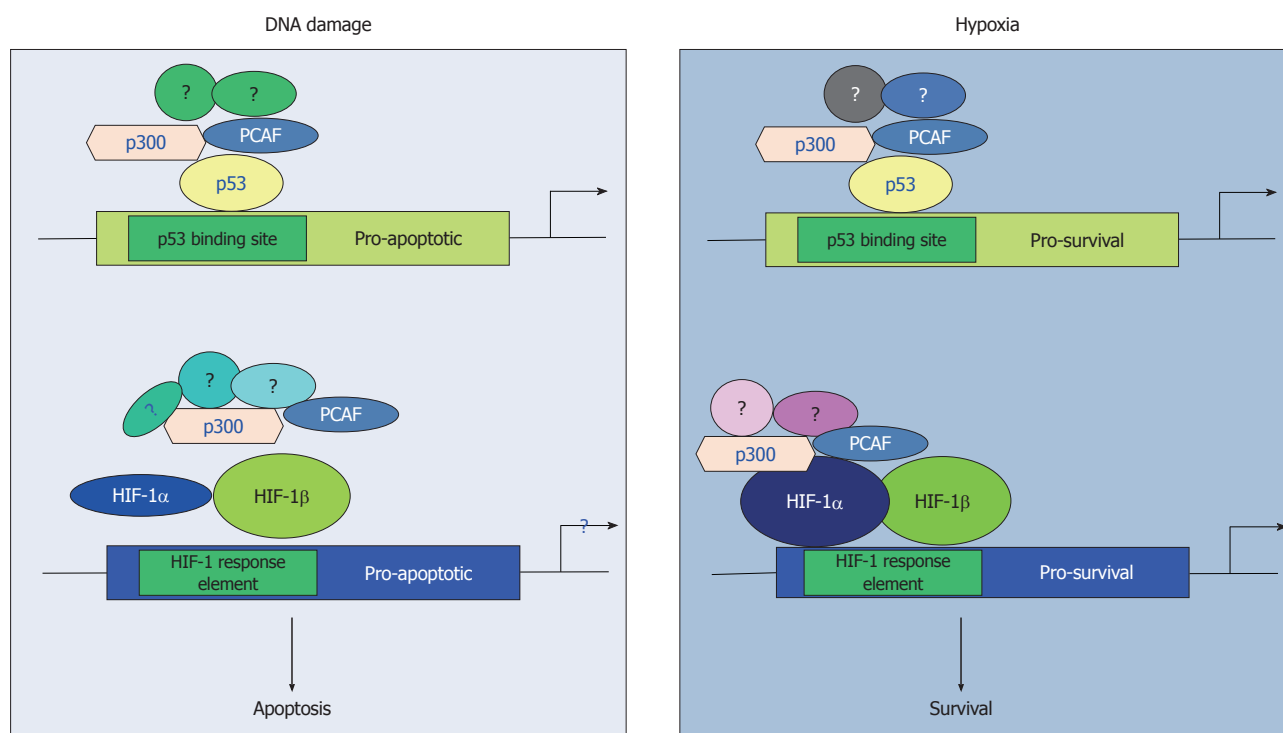


Figure 1 p300/CBP associated factor mediates p53 and hypoxia inducible factor-1 α transcription target selectivity in a manner dependent on the type of stress. In DNA damage conditions the p300/CBP associated factor (PCAF) is recruited to the promoters of pro-apoptotic gene targets thus inducing p53 mediated cell death, whereas in conditions of low oxygen availability PCAF mediates p53 and hypoxia inducible factor 1 α (HIF-1 α) post-translational modifications that selectively target both transcription factors to a subset of their transcription target genes with pro-survival activity thereby inducing cell proliferation.

and indicated that under certain conditions p53 and HIF-1 α co-operate in inducing apoptosis whereas they exert opposing functions in G1 cell cycle arrest^[54,55]. p53 has also been shown to be stabilized in hypoxia mimicking conditions in a HIF-1 α dependent manner^[56] although its transcriptional activity is attenuated in hypoxia since it is incapable to induce the expression of its transcription targets including pro-apoptotic members of the Bcl-2 family under these conditions^[57]. Although the molecular mechanisms involved have not yet been clearly elucidated, it appears that both p53 and HIF-1 α regulate cellular energy production pathways by modulating the gene expression of glucose transporters and enzymes involved in glycolysis and oxidative phosphorylation. In particular, the glucose transporter GLUT-1 is downregulated by p53 and upregulated by HIF-1 α ^[58-60] and similarly hexokinase 2 is upregulated by mutated p53^[61,62], and induced by HIF-1 α ^[63]. These contradicting observations are due at least in part to the differential interactions of p53 and HIF-1 α with their common co-activators or co-repressors^[64-67].

ROLE OF THE COFACTORS SHARED BETWEEN p53 AND HIF-1 α

The p300/CBP transcriptional coactivator assembles a number of diverse cofactor proteins into multicomponent complexes^[68] and is itself involved in the regulation of the transcriptional activity of both HIF-1 and p53^[69,70]. The steroid receptor coactivator 1 is a component of the p300/

CBP complex^[71] and another common cofactor shared between HIF-1^[72] and p53^[73]. In addition, the nuclear receptor coactivator TIF2 interacts with HIF-1 to potentiate its transcriptional activity^[74], although it inhibits p53 transcription potential when fused with the acetyltransferase MOZ associated with acute myeloid leukaemia^[75].

Our studies investigating the crosstalk between p53 and HIF-1 α ^[64,65,76] have elucidated an additional molecular mechanism explaining the inability of p53 to activate its pro-apoptotic targets in hypoxia and implicate p300/CBP associated factor (PCAF) in the fine-tuning of the transcriptional activity and protein stability of both p53 and HIF-1 α in DNA damage and hypoxic conditions. PCAF is a common cofactor for both p53 and HIF-1 α ^[64,67] and is recruited to the transcriptional complex of the one or the other transcription factor in a tissue and type of stress dependent manner (Figure 1) determining the pathway of energy production (Figure 2) and the cellular fate under diverse stress conditions^[64,65] providing an additional evidence for the importance of the co-activator function in determining the cell fate under hypoxia by modulating both p53 and HIF-1 α responses.

IMPLICATIONS ON THE EFFICACY OF ANTI-CANCER THERAPIES

The therapeutic activity of many anti-cancer agents depends on their ability to specifically and selectively induce apoptotic pathways in cancer cells. Radioactivity and

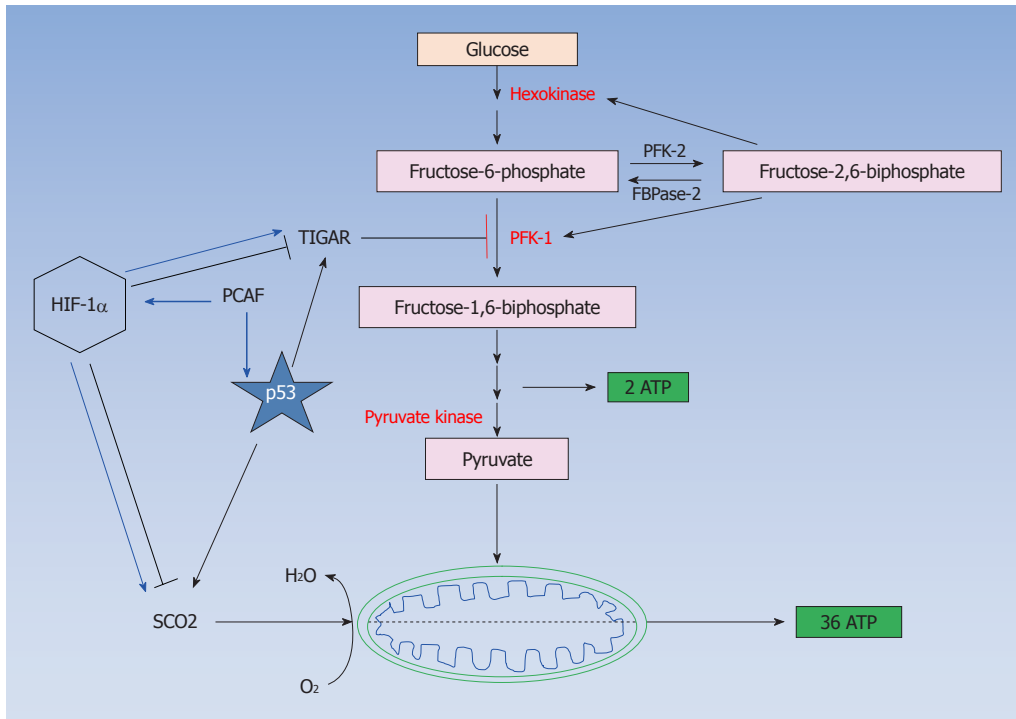


Figure 2 p300/CBP associated factor determines cellular energy metabolism pathways under diverse types of stress. Differential post-translational modifications of p53 and hypoxia inducible factor 1 α (HIF-1 α) mediated by p300/CBP associated factor (PCAF), distinctly modulate cellular energy metabolism pathways by activating or repressing the expression of their target genes Tp53 induced glycolysis and apoptosis regulator (TIGAR) and synthesis of cytochrome c oxidase 2 (SC02). PFK: Phosphofructokinase.

chemotherapeutic drugs mediate their pro-apoptotic effects through the induction of pro-apoptotic pathways regulated by transcription factors such as the tumor suppressor protein p53. The tumor suppressor p53 signalling pathway is a highly regulated process involving a cascade of events, mediated among other pathways by various transcriptional co-factors such as the p300, and other p300 associated factors such as the tetratricopeptide domain 5 and PCAF^[77,78]. These co-factors regulate the p53 transcriptional activity and protein stability by acetylating different lysine residues in its C-terminal region and in this way they contribute to the p53 mediated cellular adaptation to diverse types of stress^[79,80]. In addition, it has become clear from the studies investigating the molecular mechanisms of the regulation of HIF-1 α protein stability and transcriptional activity that p300 is required for the trans-activation of HIF-1 α and that there is competition for limiting amounts of this cofactor in hypoxia between HIF-1 α and p53^[69,81-83].

Poor response or resistance to anti-cancer chemotherapeutics by hypoxic tumors has been evidenced and it is attributed to the lack of vascular system that would allow efficient drug delivery to these tumors^[84]. Likewise, radiation therapy requires oxygen radicals for efficient production of DNA strand breaks, and thus hypoxic tumor microenvironment contributes to radioresistance^[44,84]. Furthermore, repression of the p53 transcriptional activity and inability of this transcription factor to induce its pro-apoptotic targets in hypoxic conditions is an additional mechanism conferring drug resistance to

hypoxic tumors^[85].

CONCLUSION AND FUTURE DIRECTIONS

Our observations have provided evidence supporting the view that distinct subpopulations of transcription co-activator complexes as well as differential posttranslational modifications determine the transcriptional target selectivity of both p53 and HIF-1 α under diverse micro-environmental conditions^[64,65,76] resulting in the expression of distinct subsets of genes, which carry out different functions, in a type of stress dependent manner. This distinction in the transcriptional cofactors' function can be interpreted in a variety of ways. Firstly, transcription cofactors might facilitate the recruitment of different transcription factors to distinct regions of the genome^[86] thus allowing different transcription factors to carry out specialised functions determining the cellular fate (survival or apoptosis) (Figure 1). Secondly, differences in the structure of the promoter between the different targets of various transcription factors could be responsible for preferential binding of particular subsets of these targets by alternatively posttranslationally modified transcription factors. For example, PCAF dependent acetylation of either p53 or HIF-1 α is a mechanism by which these transcription factors distinguish between their pro-survival or pro-apoptotic target promoters^[64] or glycolytic or oxidative phosphorylation inducers (Figure 2)^[65,87-89].

To substantiate this hypothesis we are currently using genome wide ChIP-seq approaches to uncover the spe-

cific transcriptional circuitries that determine the specificity and target selectivity of several transcription factors including p53, glucocorticoid receptor, estrogen receptor, HIF-1 α and NF- κ B which play very important roles in carcinogenesis. The ultimate aim of this investigation is to acquire essential knowledge that will guide the identification of new transcriptional targets in the DNA damage response and low oxygen availability networks and thus facilitate the development of selective therapeutics for potential personalized cancer therapeutics.

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P- Reviewer: Maluf SW S- Editor: Zhai HH
L- Editor: A E- Editor: Zheng XM



An inhibitor of HIF- α subunit expression suppresses hypoxia-induced dedifferentiation of human NSCLC into cancer stem cell-like cells

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Supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, (No. 18590280); the Foundation for the Promotion of Cancer Research in Japan; Grant-in-Aid for Japan Arteriosclerosis Research Foundation; and Shimane University "S-TAKUMI Medical Nanotechnology" Project

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Received: June 17, 2013 Revised: August 29, 2013

Accepted: September 3, 2013

Published online: November 27, 2013

Abstract

AIM: To investigate whether hypoxia induces dedifferentiation of non-small cell lung cancer (NSCLC) cells and whether a hypoxia-inducible factor (HIF) inhibitor is able to suppress the process.

METHODS: Human lung adenocarcinoma A549 cells and squamous carcinoma QG56 cells were cultured under normoxic (21% O₂) or hypoxic (4% or 1% O₂) conditions. The expression of the following genes were examined by

reverse transcription-polymerase chain reaction, Western blotting and/or immunofluorescence: HIF-1 α and HIF-2 α subunits; differentiation marker genes, namely surfactant protein C (*SP-C*) (type II alveolar cell marker), *CC10* (type I alveolar cell marker) and aquaporin 5 (*AQP5*) (Clara cell marker); and stem cell-associated genes, namely *CD133*, *OCT4*, and *Musashi-1* (*MSI1*). The tumor sphere-forming ability of the cells was evaluated by culturing them in serum-free growth factor-rich medium containing epidermal growth factor (EGF) and fibroblast growth factor (FGF). *CD133* expression in hypoxic regions in A549 tumors was examined by double-immunostaining of tissue cryosections with an anti-2-nitroimidazole EF5 antibody and an anti-*CD133* antibody. The metastatic ability of A549 cells was examined macroscopically and histologically after injecting them into the tail vein of immunocompromised mice.

RESULTS: A549 cells primarily expressed *SP-C*, and QG56 cells expressed *CC10* and *AQP5*. Exposure of A549 cells to hypoxia resulted in a marked down-regulation of *SP-C* and upregulation of *CD133*, *OCT4*, and *MSI1* in a time-dependent manner. Moreover, hypoxia mimetics, namely desferrioxamine and cobalt chloride, elicited similar effects. Ectopic expression of the constitutively active HIF-1 α subunit also caused the downregulation of *SP-C* and upregulation of *CD133* and *MSI1* but not *OCT4*, which is a direct target of HIF-2. Hypoxia enhanced the sphere-forming activity of A549 cells in serum-free medium containing EGF and FGF. Similarly, hypoxia downregulated the expression of *CC10* and *AQP5* genes and upregulated *CD133*, *OCT4*, and *MSI1* genes in QG56 cells. TX-402 (3-amino-2-quinolinecarbonitrile 1, 4-dioxide), which is a small molecule inhibitor of the expression of HIF-1 α and HIF-2 α subunits under hypoxic conditions, inhibited the upregulation of *SP-C* and hypoxia-induced down-regulation of *CD133*, *OCT4*, and *MSI1*. Notably, TX-402 significantly suppressed the hypoxia-enhanced lung-colonizing abil-

ity of A549 cells.

CONCLUSION: Hypoxia induces the de-differentiation of NSCLC cells into cancer stem cell-like cells, and HIF inhibitors are promising agents to prevent this process.

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Key words: Non-small cell lung cancer; Tumor micro-environment; Hypoxia; Hypoxia-inducible factor; Differentiation; Cancer stem cells; Hypoxia-inducible factor inhibitor

Core tip: Hypoxia induces the de-differentiation of human non-small cell lung cancer cells into cancer stem cell-like cells, and TX-402, a small-molecule inhibitor of hypoxia-inducible factor (HIF)-1 α and HIF-2 α expression, suppresses this hypoxia-induced process and, importantly, the metastatic ability of the cells.

Akimoto M, Nagasawa H, Hori H, Uto Y, Honma Y, Takenaga K. An inhibitor of HIF- α subunit expression suppresses hypoxia-induced dedifferentiation of human NSCLC into cancer stem cell-like cells. *World J Med Genet* 2013; 3(4): 41-54 Available from: URL: <http://www.wjgnet.com/2220-3184/full/v3/i4/41.htm> DOI: <http://dx.doi.org/10.5496/wjmg.v3.i4.41>

INTRODUCTION

In most solid tumors, hypoxic regions are generated because of a shortage in oxygen supply^[1-3]. Hypoxia influences many aspects of cancer cell biology, including neoangiogenesis, energy metabolism, cell survival, radiosensitivity, chemosensitivity, differentiation and invasion/metastasis^[1-4]. Thus, hypoxia correlates with poor patient outcomes^[5]. Recent preclinical studies have also demonstrated that anti-angiogenic therapies generate intratumoral hypoxia and, thereby, elicit increases in invasiveness and metastasis^[6-8].

Hypoxia activates the expression of hundreds of genes in each cell^[1-3]. Many of these genes are regulated by hypoxia-inducible factors (HIFs) such as HIF-1 and HIF-2. Under normoxic conditions, the α subunit of HIF-1 (HIF-1 α) is hydroxylated at the proline-402 and proline-564 residues by specific Fe²⁺, 2-oxoglutarate, and O₂-dependent prolyl hydroxylases. The subunit is then recognized and ubiquitinated by the von Hippel-Lindau tumor suppressor protein complex, leading to degradation *via* the ubiquitin-proteasome pathway. Under hypoxic conditions, the HIF- α subunit stabilizes and dimerizes with the HIF- β subunit. In the nucleus, HIF-1 binds to the hypoxia response element (HRE) of hypoxia-inducible genes and transactivates their transcription^[9]. Chelating or substituting Fe²⁺ with desferrioxamine (DFO) or CoCl₂, respectively, increases the levels of HIF-1 α and HIF-2 α ^[1-3]. Although there are many similarities between HIF-1 and HIF-2, each has common as well as different HRE target genes.

Hypoxia influences stem cell self-renewal and multipotency^[10]. HIF-2 but not HIF-1 is reported to regulate the expression of OCT4, a POU transcription factor that is associated with the self-renewal and pluripotency of stem cells^[11]. Hypoxia is also known to induce the dedifferentiation of neuroblastoma and breast cancer cells^[12]. These data indicate that hypoxia is involved not only in the de-differentiation of tumor cells but also in the maintenance of cancer stem cells (CSCs) with high metastatic potential.

The lung is composed of multiple types of cells such as Clara, alveolar type I (AT1), alveolar type II (AT2), and pulmonary neuroendocrine cells^[13]. Each cell type expresses lineage-specific differentiation marker gene products. For example, CC10 (also known as CCA or CCSP), aquaporin 5 (AQP5), and pro-surfactant protein C (SP-C) are the specific markers for Clara, AT1 and AT2 cells, respectively^[14-16]. A rare population of progenitor cells, referred to as the bronchoalveolar stem cells (BASCs), exists in the bronchoalveolar duct junction of mouse normal lung^[17]. Lung adenocarcinoma is thought to originate from BASCs, which frequently co-express SP-C and CC10^[17-19]. Several recent studies have demonstrated the existence of a rare population of CD133-positive undifferentiated cells in small cell and non-small cell lung cancer (NSCLC) that exhibit CSC characteristics^[20-23]. The metabolic enzyme, glycine decarboxylase (GLDC), has also been reported to be a key characteristic of CSCs in NSCLC^[24]. In contrast, it is largely unknown how tumor hypoxia affects the differentiation- and stem cell-related gene expression in NSCLC cells.

Many small molecule HIF-1 inhibitors have been identified or developed to inhibit angiogenesis and suppress the growth of a variety of tumor cells^[25]. However, the influence of such HIF-1 inhibitors on the hypoxia-induced dedifferentiation of human lung cancer cells to CSC-like cells remains unknown. Assuming that HIF-1 α and HIF-2 α are necessary for inducing and maintaining CSC characteristics under hypoxic conditions, inhibitors of their expression or activity would have a profound inhibitory effect on such hypoxia-induced processes.

TX-402 is a member of a group of hypoxia-selective cytotoxins, which includes tirapazamine (TPZ), that are activated by bioreduction to selectively exhibit cytotoxicity under severe hypoxia (< 1% O₂). TX-402 and its analogs inhibit HIF-1 α protein synthesis under hypoxia without affecting the HIF-1 α steady-state mRNA level and the protein degradation rate, and they also reduce the hypoxia-inducible expression of vascular endothelial growth factor (VEGF) and angiogenesis^[26-28]. Although the mechanism by which TX-402 inhibits HIF-1 α protein synthesis is still unclear, TPZ and other hypoxic cytotoxins have recently been reported to inhibit HIF-1 α translation through the phosphorylation of translation initiation factor 2 α (eIF2 α) and/or the dephosphorylation of mTOR and 4E-BP1^[29,30]. Of interest, TPZ has been shown to selectively deplete primitive hematopoietic stem cell subsets in bone marrow^[31]. Therefore, this class of bioreductive agents could have a major impact on cancer therapy if developed appropriately. Against this background,

Table 1 Primers used for reverse transcription-polymerase chain reaction analyses

Gene	Forward primer	Reverse primer	Accession #
CC10	CTTTCAGCGTGCATCGAAA	TTGAAGAGAGCAAGGCTGGT	U01101
SP-C	TCCACATGAGCCAGAAACAC	CTGGCCAGCTTAGACGTAG	NM_003018
AQP5	CTACTTCACTGGCTGCTCCA	GTGGTCAGCTCCATGGTCTT	NM_001651
CD133	TGGAGTGCAGTAACATGAG	TGCACATGAAAAGACCTGGG	NM_006017
4-Oct	GAGGAGTCCCAGGACATCAA	CTCCAGGTTGCCTCTCACTC	NM_002701
MSI1	GTTTCAGAGCGTTGGACCTTC	AAACCCAAAACACGAACAGC	NM_002442
GLDC	ATTCTCGTTGATCCCCGTGTC	GCGATGTCTACCCCAAATTCTC	NM_000170
NANOG	ACCAGACCCAGAACATCCAG	TTCACACGCTTTCAGGTTGC	NM_024865
NES	AACAGCGACGGAGGTCTCTA	TTCTTGTGCCCGCAGACTT	NM_006617
BMI1	AGAGCTGGAAGTCGAGTGT	GCACCTTCACATTCCTCTC	NM_005180
EGFR	GCACGAGTAACAAGCTCACG	TTCTCTGATGATCTGCAGG	NM_005228
ERBB2	AGCAGAGGATGGAACACAGCGG	CTCCTGGATATTGGCACTGG	NM_004448
NOTCH1	TGCTGGACGAGTACAACCTG	CGCATTGACCAATCAAACCTG	AF308602
NOTCH2	ACCCTTGTGAGAATGCTGCT	CCATACCACTGAAGCCTGGT	NM_024408
CD117	CTATGCTCTCGCACCTTTCC	CAATGAAGTGCCCTGAAGT	X06182
MET	GGTTTTTCTGTGGCTGAAA	GGCATGAACCGTTCTGAGAT	NM_001127500
CD34	ACAACACGTGGTGGCTGATA	GAGTTTACCTGCCCCTCCTC	NM_001773
CD44	TGGAGCAAACACAACCTCTG	TCCACTTGGCTTTCTGTCTC	NM_000610
CD45	AGATGCCCAGTGTTCAC	AGGGTTGAGTTTTCATTTGG	NM_002838
PCGF2	TTCAGTGGAAACITTTGTC	AGGTGAGACTCCACCACAG	NM_007144
CDH1	GGTTATTCCTCCCATCAGCT	CTTGGCTGAGGATGGTGTA	L08599
SOX2	CCCCCTGTGGTTACCTCTTC	TTCTCCCCCTCC AGTTTCG-3	NM_003106
RUNX3	GCTGTTATGCGTATTCCTGAG	TGAAGTGGCTGTGGTGCTGAGTGA	NM_001031680
ACTB	TGACGGGGTCACCCACACTGTGCCATCTA	CTAGAAGCATTTGCGGTGGACGATGGAGGG	NM_001101

GLDC: Glycine decarboxylase.

in the present study, we primarily employed human lung adenocarcinoma A549 cells and investigated the effects of hypoxia and TX-402 on differentiation- and stem cell-related gene expression and metastasis.

MATERIALS AND METHODS

Cells and cell culture

Human lung adenocarcinoma cells (A549, PC3, PC9, and PC14), squamous cell carcinoma cells (PC1, PC10, and QG56), bronchoalveolar carcinoma cells (H358), and small cell carcinoma cells (PC6 and QG90) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum supplemented with penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified atmosphere with 21% O₂/5% CO₂ (normoxia) or 1% O₂/5% CO₂ (hypoxia) unless otherwise stated. In some experiments, the cells were cultured in 4% O₂/5% CO₂. The cells were provided by the Chiba Cancer Center Research Institute^[32].

Reagents

TX-402 (3-amino-2-quinoxalinecarbonitrile 1,4-dioxide) was synthesized according to a previous report^[33]. DFO and cobalt chloride were obtained from Sigma-Aldrich (St. Louis, MO, United States). EF5 was provided by the National Cancer Institute (CTEP).

Antibodies

Mouse monoclonal anti-β-actin antibody was obtained from Sigma-Aldrich. Mouse monoclonal anti-HIF-1α and rabbit polyclonal anti-HIF-2α antibodies were purchased

from Novus Biologicals (Littleton, CO, United States). The rabbit polyclonal anti-CD133 antibody, goat polyclonal anti-OCT4 antibody and rabbit polyclonal anti-E2F-1 antibody were supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States), and the rabbit polyclonal anti-pro-SP-C antibody and phycoerythrin-conjugated mouse monoclonal CD133/2 antibody were obtained from Merck Millipore (Billerica, MA, United States) and Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), respectively. The goat anti-Musashi-1 (MSI1) antibody was obtained from R and D Systems (McKinley Place, MN, United States). The Cy3-labeled mouse monoclonal anti-EF5 antibody (ELK3-51) was kindly provided by Dr. Koch at Pennsylvania State University.

Semiquantitative reverse transcription polymerase chain reaction

Reverse transcription (RT) was performed in a 10 µL reaction mixture containing 1 µg of total RNA, which was extracted with guanidinium thiocyanate, 250 ng of oligo(dT), and 100 units of murine leukemia virus reverse transcriptase (Life Technologies, Carlsbad, CA, United States) for 1 h at 37 °C. The resulting cDNA was used to amplify target cDNAs using *G*oTaq DNA polymerase (Agilent Technologies, Santa Clara, CA, United States). The sense and antisense primers used for polymerase chain reaction (PCR) are listed in Table 1. The PCR conditions were as follows: 95 °C for 5 min; 25–35 cycles at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; and 72 °C for 7 min. β-Actin was used as a loading control, which was run for each sample under the same conditions as those used for the other genes, except that the

number of cycles was 23–25. PCR products were separated on a 1.2% agarose gel. Quantification of the density of each band was performed using ImageJ version 1.45 software (National Institutes of Health).

Transfection of the plasmid encoding constitutively active HIF-1 α

To express constitutively active HIF-1 α , A549 cells were transfected with pcDNA3.1 as a control and pcDNA3.1/HIF-1 α ^{P402A/P564A} using Lipofectamine 2000 (Life Technologies). Two days after transfection, total RNA was prepared as described above and used for RT-PCR analysis.

Western blotting analyses

Cells were lysed in 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L PMSF, and protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were centrifuged at 15000 *g* for 10 min at 4 °C, and the supernatants were used for detecting SP-C and CD133. Nuclear extracts were prepared by using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, United States) and were then used for detecting HIFs, OCT4 and MSI1. Proteins were resolved by 10% or 15% SDS-PAGE gels and transferred to an Immobilon-P membrane (Merck Millipore). The membrane was blocked with 5% dry milk in TBS-T and incubated with anti-HIF-1 α , anti-HIF-2 α , anti-SP-C, anti-CD133, anti-OCT4, anti-MSI1 or anti-E2F-1 antibodies followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were detected using ECL Western blotting detection reagents (GE Healthcare, Waukesha, WI, United States).

Tumor spheroid (pneumosphere) formation

To evaluate the effect of hypoxia on pneumosphere formation, cells pre-cultured under 4% O₂ for 4 d were suspended at a density of 2000 cells/mL in BEBM (bronchial epithelial cell growth) medium (Lonza, Walkersville, MD, United States) supplemented with SingleQuots, which contains retinoic acid, bovine pituitary extract, insulin, hydrocortisone, transferrin, triiodothyronine, epinephrine, gentamicin, and amphotericin B (Lonza), as well as 10 ng/mL human epidermal growth factor (EGF) (PeproTech Inc., NJ, United States), and 10 ng/mL human basic fibroblast growth factor (bFGF) (PeproTech)^[34]. Cells were incubated in PrimeSurface culture dishes (35 mm) (Sumitomo Bakelite Co., Tokyo, Japan) under normoxic or hypoxic (4% O₂) conditions for 10 d. EGF and bFGF were added every 3 d. The size of the spheres was calculated by the following equation: size (μ m) = (a + b)/2; where a and b are the larger and smaller diameters, respectively.

Colony formation assay

The survival of A549 cells treated with TX-402 under normoxic or hypoxic conditions was determined in a colony formation assay. For this assay, A549 cells dissociating to single cells were seeded in a 6-well plate at a density of 5×10^4 cells per well in 2 mL of medium. Af-

ter attachment, the cells were cultured under normoxic or hypoxic conditions in the presence of DMSO (0.1%) or TX-402 (final concentration of 20 μ mol/L) for 3 d. After the treatment, viable cells were counted and seeded in a 10-cm culture dish at a concentration of 100 cells/dish. The cells were further cultured for 14 d under normoxic conditions, and the colonies that were formed were fixed with methanol, stained with 0.05% crystal violet, and counted.

Immunocytochemistry

A549 cells on cover slips were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed for 30 min with 4% formaldehyde and 5% sucrose in DPBS. After washing with DPBS, the cells were permeabilized in 0.5% Triton X-100 in DPBS for 10 min followed by washing three times with DPBS. In some experiments, this permeabilization step was omitted. The cells were then treated with 3% bovine serum albumin (BSA) in DPBS containing 0.1% glycine for 1 h to block nonspecific binding sites. After extensive washing, the cells were incubated with anti-SP-C, anti-CD133, anti-OCT4 or anti-MSI1 antibodies in DPBS containing 1 mg/mL BSA and 0.1% (v/v) normal rabbit serum, normal goat serum or normal chicken serum depending on the secondary antibody at 4 °C overnight. Primary antibodies were omitted for negative control studies. After washing with DPBS, the first antibodies were localized with the appropriate secondary antibodies. The secondary antibodies used were FITC-goat anti-rabbit IgG, TRITC-goat anti-rabbit IgG, Alexa Fluor 488-goat anti-rabbit IgG, and Alexa Fluor 488-chicken anti-goat IgG. After rinsing, the cells were counterstained with DAPI (1 μ g/mL) in DPBS, and the coverslips were mounted in 50% glycerol in DPBS containing 1 mg/mL *p*-phenylenediamine to inhibit photobleaching. The cells were observed under a confocal laser microscope (Fluoview, Olympus, Tokyo, Japan).

Immunohistochemistry

All animal experiments were performed in compliance with the institutional guidelines. A549 cells (5×10^6 cells) were inoculated subcutaneously into Balb/c nude mice. When an estimated tumor volume reached approximately 500 mm³, 300 μ L of EF5 solution (3 mg/mL) was administered intraperitoneally into the mice. Two hours later, subcutaneous tumors were surgically removed and frozen in optimum cutting temperature compound. Cryostat sections cut at a thickness of 10 μ m were fixed with 4% paraformaldehyde and washed with DPBS. For the detection of CD133, tissue samples were treated with 5% donkey serum in DPBS/1% (w/v) BSA/20% (w/v) dry milk for 1 h to block nonspecific binding sites. Sections were then rinsed with DPBS and incubated with an anti-CD133 antibody overnight at 4 °C. After extensive washing with DPBS, the sections were incubated with a FITC-labeled donkey anti-goat secondary antibody for 1 h. After fixation with 4% formaldehyde, the sections were washed with DPBS, treated with 5% mouse serum

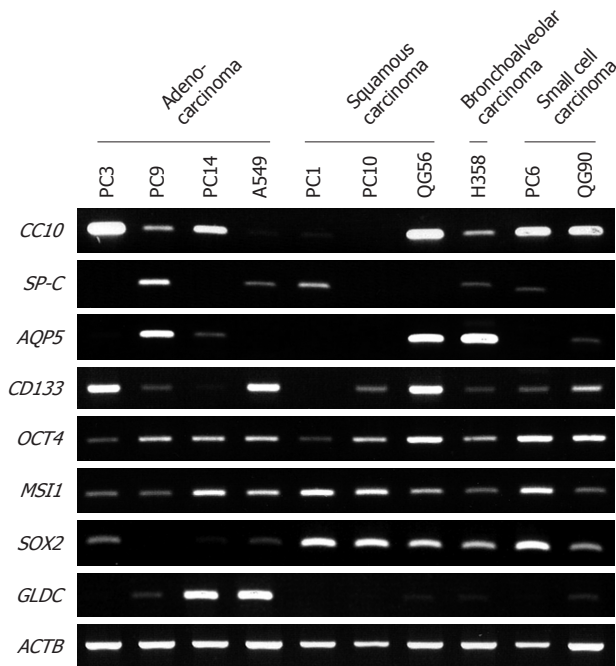


Figure 1 Expression of lineage-specific differentiation- and stem cell-associated genes in various lung cancer cell lines. Total RNA isolated from the cells was subjected to reverse transcription-polymerase chain reaction analyses; GLDC: Glycine decarboxylase; MSI1: Musashi-1; ACTB: β -Actin.

in DPBS/20% (w/v) dry milk/0.3% (v/v) Tween 20 overnight at 4 °C, rinsed with 0.3% Tween 20 in DPBS, and then incubated with Cy3-labeled anti-EF5 antibody (ELK3-51) at 4 °C overnight to detect hypoxic cells. Tissue sections were counterstained with DAPI and observed under a confocal laser microscope (Fluoview, Olympus).

Analysis of lung-colonizing potential

For the lung-colonizing assay, A549 cells (2×10^5 cells) cultured under normoxic or hypoxic (1% O_2) conditions for 3 or 5 d were injected into the tail vein of 6-wk-old Balb/c nude mice (CLEA Japan, Inc.). The lungs were removed 40 d later and fixed in Bouin's solution, and the parietal nodules were counted. For histology, formalin-fixed, paraffin-embedded lung tissues were sectioned at a thickness of 5 μ m, mounted, and stained with hematoxylin and eosin.

Statistical analysis

The Mann-Whitney *U* test and Student's *t*-test were used to determine statistical significance in metastasis assays and other assays, respectively. *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression of lineage-specific differentiation marker genes and stem cell-related genes in different types of human lung cancer cell lines

We examined the expression of lineage-specific differ-

entiation marker genes for Clara (*CC10*), AT1 (*AQP5*), and AT2 (*SP-C*) cells as well as several stem cell-related genes (*CD133*, *OCT4*, *MSI1*, *SOX2* and *GLDC*) in various types of lung cancer cell lines (Figure 1). Among the adenocarcinoma cell lines, PC3 and PC14 cells expressed *CC10*, and A549 cells primarily expressed *SP-C*. PC9 cells expressed *CC10*, *SP-C*, and *AQP5*. Among the squamous carcinoma cell lines, *SP-C* was detected only in PC1 cells. QG56 cells were positive for *CC10* and *AQP5*, but PC10 cells expressed none of the markers. By comparison, bronchoalveolar carcinoma H358 cells expressed all of the lineage-specific differentiation marker genes. Small cell carcinoma PC6 and QG90 cells expressed high levels of *CC10*. The stem cell-related gene, *CD133*, was expressed at higher levels in PC3, A549 and QG56 cells compared to the other cells. All cell lines were *OCT4*-positive/*MSI1*-positive. The expression of *SOX2* was detected in all cell lines except PC9 cells. *GLDC* was highly expressed in PC14 and A549 cells compared with other cell lines. Taken together, these results suggest that there is not a simple causal relationship between the expression of the differentiation marker genes and the expression of the stem cell-related genes. For subsequent analyses, we mostly used A549 and QG56 cells.

Hypoxia suppresses the expression of lineage-specific marker genes

We first confirmed the expression of HIF-1 α and HIF-2 α in A549 and QG56 cells at the mRNA level (Figure 2A). We then cultured these cells under normoxic or hypoxic conditions for up to 5 d and monitored cell growth and viability. The growth of A549 cells under hypoxic conditions was slightly inhibited by 4 d after the onset of hypoxia compared with their growth under normoxic conditions (Figure 2B). No cell death was observed in hypoxic conditions (Figure 2D). Hypoxia also slightly inhibited the growth of QG56 cells but had no detectable effect on viability (Figure 2C, D). Because these hypoxic conditions did not appear to be cytotoxic, we cultured A549 and QG56 cells under hypoxia, and we then examined the expression levels of *SP-C*, *CC10*, and *AQP5*. As described above, normoxic A549 cells primarily expressed *SP-C* and small amounts of *CC10* and *AQP5* (Figure 3A). Upon hypoxic exposure, the expression level of these genes was greatly reduced in a time-dependent manner (Figure 3A, B, D). QG56 cells mainly expressed *CC10* and *AQP5* (Figure 3A), and the expression level of these genes was greatly reduced in a time-dependent manner after exposure to hypoxia (Figure 3A, C, E).

Hypoxia influences the expression of stem cell-related genes

The above results prompted us to compare in detail the expression of a panel of stem cell-related genes between normoxic and hypoxic cells. Among the genes tested, the expression levels of *CD133*, *OCT4*, and *MSI1* were increased by hypoxia in A549 cells (Figure 4A left, B, C) in a time-dependent manner (Figure 4F). *NANOG* and

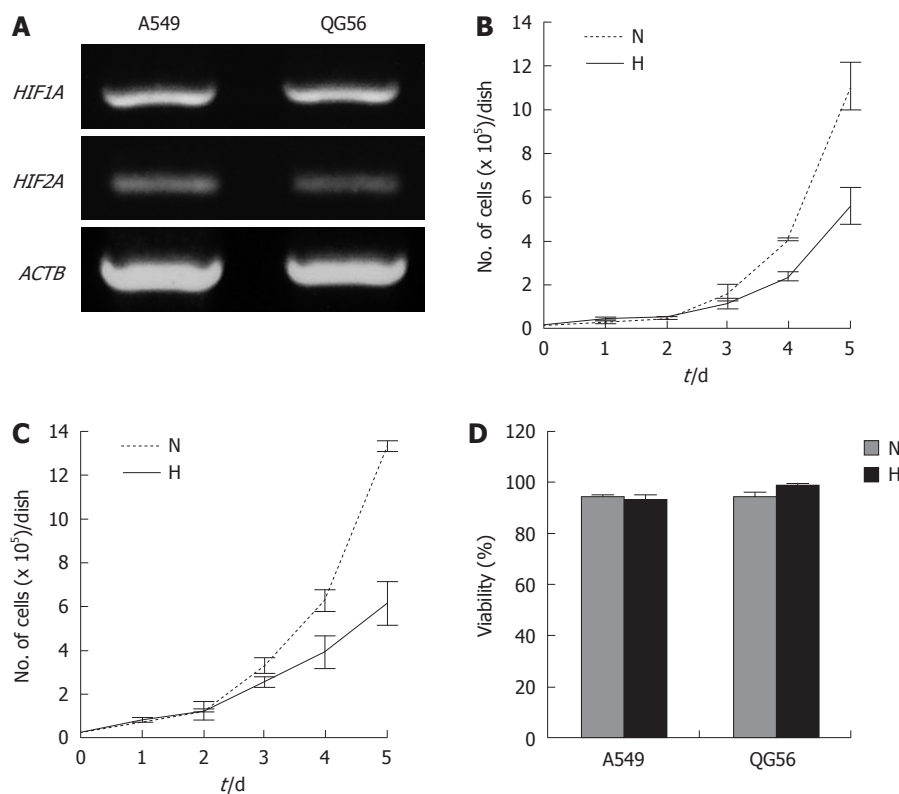


Figure 2 Expression of hypoxia-inducible factor α subunits and cell growth in A549 and QG56 cells under hypoxic conditions. The cells were cultured under normoxic (N) or hypoxic (H) conditions. A: Expression of hypoxia-inducible factor (HIF)-1 α and HIF-2 α mRNA; B: Cell growth of A549; C: Cell growth of QG56 cells; D: Cell viability; The cells were cultured for 5 d; Bars, SD ($n = 3$).

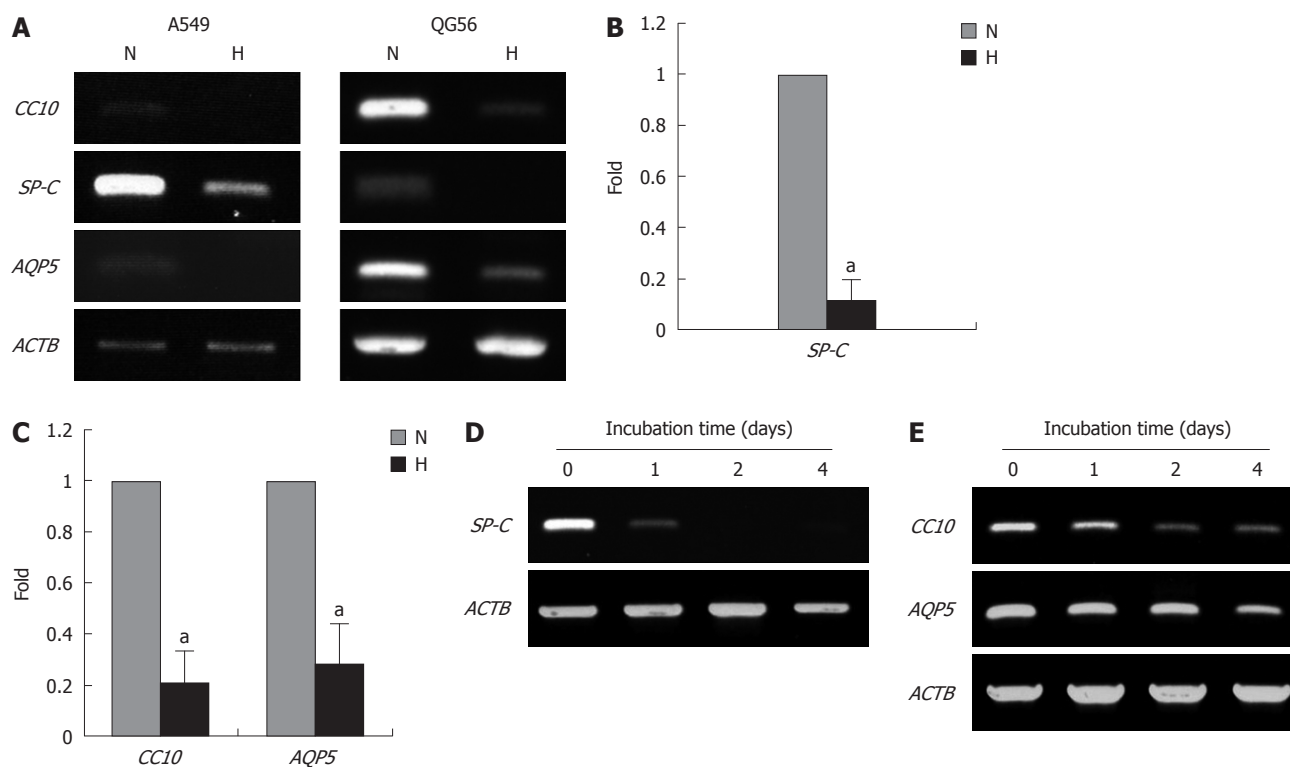


Figure 3 Effect of hypoxia on the expression of differentiation-associated genes. The cells were cultured under normoxic (N) or hypoxic (H) conditions for 5 d. A: Expression of differentiation-associated genes in A549 and QG56 cells; B: Normalized expression levels of the genes in QG56 cells; C: Normalized expression levels of the genes in A549 cells; D: Time-course of the expression of *SP-C* in A549 cells; E: The expression of *CC10* and *AQP5* in QG56 cells; The expression level of each gene was normalized to that of β -Actin (*ACTB*); Data are shown as fold-change relative to normoxia (normoxia values set to equal 1), $^aP < 0.05$ ($n = 3$); The cells were cultured under hypoxic conditions for the indicated periods.

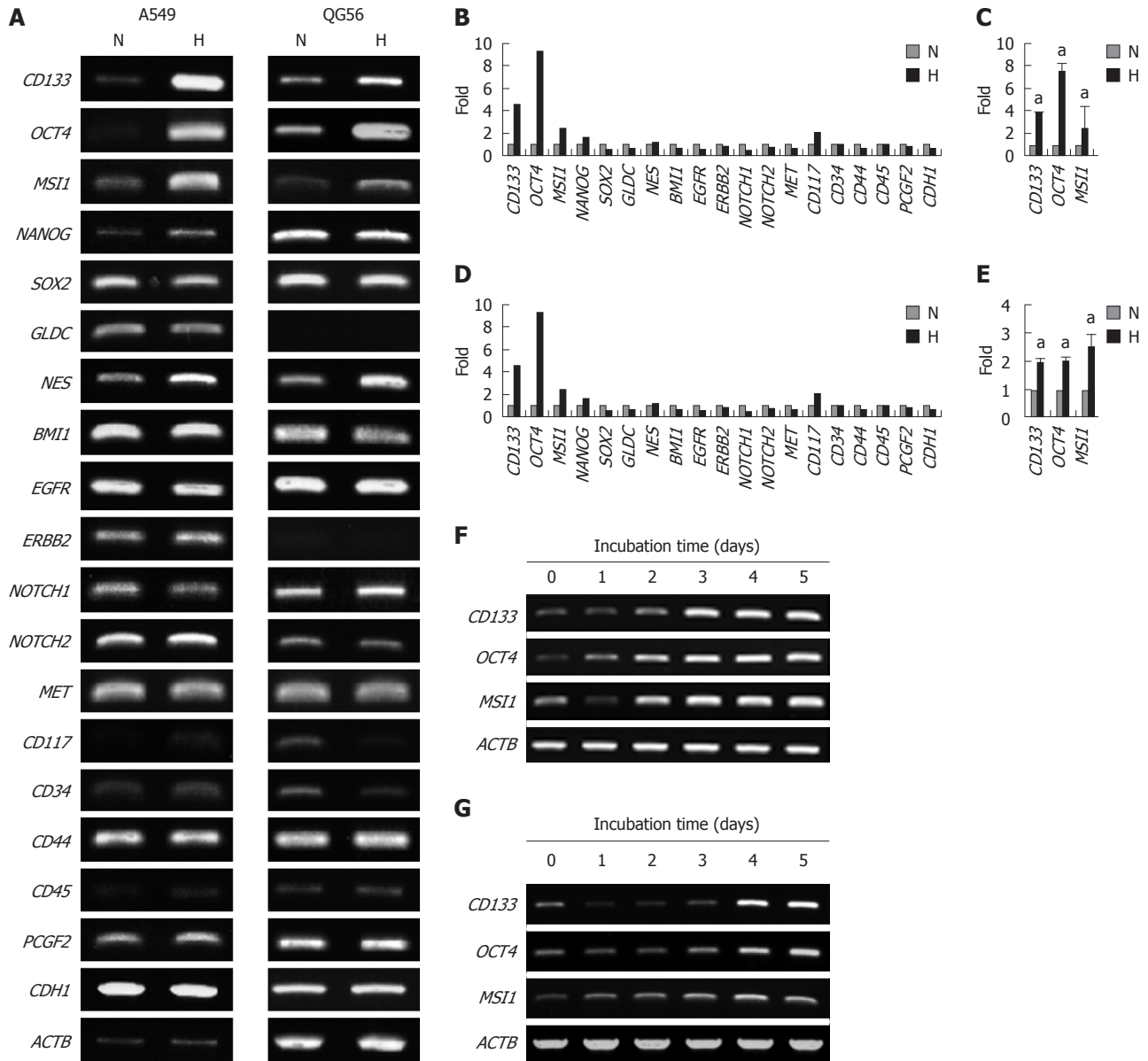


Figure 4 Effect of hypoxia on the expression of stem cell-associated genes. The cells were cultured under normoxic (N) or hypoxic (H) conditions. **A:** Expression of stem cell-associated genes in A549 and QG56 cells; **B and C:** Normalized expression levels of the genes in A549 cells; Cells were cultured for 5 d; **D and E:** Normalized expression levels of the genes in QG56 cells; **F:** Time-course of the expression of CD133, OCT4, and MSI1 in A549; **G:** Time-course of the expression of CD133, OCT4, and MSI1 in QG56 cells (G). The expression level of each gene was normalized to that of β -Actin (ACTB); Data are shown as fold-change relative to normoxia (normoxia values set to equal 1). $^aP < 0.05$ ($n = 3$); The cells were cultured for the indicated periods.

CD117 were also slightly upregulated by hypoxia. The expression of other genes, including *SOX2*, *GLDC*, *NES*, *BMI1*, *EGFR*, *ERBB2*, *NOTCH1*, *NOTCH2*, *CD34*, *CD44*, *CD45*, *PCGF2* (*MEL18*), and *CDH1* (*E-cadherin*), was detected in A549 cells and was only marginally affected by hypoxia (Figure 4A, B). Hypoxia also enhanced the expression of *CD133*, *OCT4*, and *MSI1* in QG56 cells, but to a lesser extent than in A549 cells (Figure 4A, D, E). Time-course experiments revealed that the expression of *OCT4*, *CD133*, and *MSI1* gradually increased under hypoxia (Figure 4G). Unlike in A549 cells, *NANOG* expression was already high in QG56 cells and was not affected by hypoxia. The expression of *NES* was slightly upregulated by hypoxia, but the expression of *SOX2*,

BMI1, *EGFR*, *ERBB2*, *NOTCH1*, *NOTCH2*, *CD34*, *CD44*, *CD45*, *PCGF2*, and *CDH1* was unaffected. The expression level of *GLDC* was quite low in these cells (Figure 4A, D).

Pneumosphere formation under normoxic and hypoxic conditions

The ability to form spheres in serum-free culture conditions has been considered to be an important marker that represents the subset population of CSC-like cells^[35]. We tested the ability of A549 and QG56 cells to form spheres under normoxic and hypoxic conditions. In this experiment, we allowed the cells to form spheres in 4% O₂ to minimize the inhibitory effect of hypoxia (1% O₂)

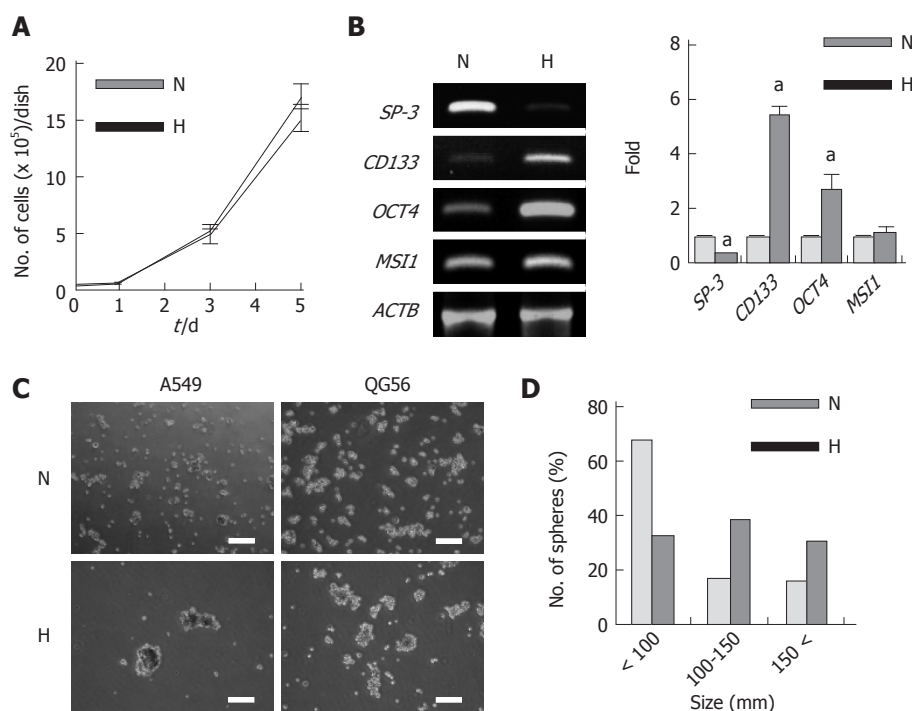


Figure 5 Changes in gene expression and sphere-forming activity of A549 and QG56 cells under hypoxic conditions. The cells were cultured under normoxic (N) or hypoxic (4% O₂) (H) conditions. **A:** Growth of A549 cells. Bars, SD ($n = 3$); **B:** Differentiation- and stem cell-related gene expression in A549 cells, the cells were cultured for 5 d, data are shown as fold-change relative to normoxia (normoxia values set to equal 1), $^aP < 0.05$ ($n = 3$); **C:** Sphere formation; A549 or QG56 cells pre-cultured under normoxic or hypoxic (1% O₂) conditions for 2 d were reseeded in BEBM supplemented with various additives (see Materials and Methods) and then further cultured for 7 d; Left panels represent A549 cells and right panels represent QG56 cells. Scale bars = 200 μ m; **D:** Size distribution of the spheres formed by A549 cells.

on cell growth. Cell growth in complete medium was not inhibited in 4% O₂ (Figure 5A) and the changes in the expression of *SP-C*, *CD133*, *OCT4*, and *MSI1* were similar to those observed in 1% O₂ (Figure 5B). The ability of A549 cells to form spheres was markedly increased under hypoxic conditions. Hypoxic cells formed larger spheres than normoxic cells (Figure 5C left, D). QG56 cells formed only loosely attached cell aggregates under normoxic conditions, but they formed more compact aggregates under hypoxic conditions (Figure 5C right). Taken together with the gene expression results, these data suggest that hypoxia can strongly induce the dedifferentiation of A549 cells but can only weakly induce the dedifferentiation of QG56 cells.

Effect of hypoxia on SP-C and CD133 protein expression in A549 cells

We examined SP-C and CD133 protein expression in A549 cells to examine the effect of hypoxia on a representative differentiation marker and a stem cell-associated marker, respectively. Immunofluorescence studies revealed that SP-C expression in normoxic A549 cells (some of which exhibited plasma membrane staining as indicated by arrowheads in Figure 6A), was significantly reduced in hypoxic A549 cells (Figure 6A). In contrast, CD133 expression was enhanced in hypoxic cells compared to normoxic cells (Figure 6B). These results were also confirmed by immunoblot analysis (Figure 6C, D). To examine whether the up-regulation of CD133 was located in hypoxic re-

gions in tumors, we immunostained cryosections prepared from A549 subcutaneous tumors with an anti-CD133 antibody. To detect hypoxic cells in tumors, we injected EF5 intraperitoneally into tumor-bearing mice 2 h before the surgical removal of the tumor masses. Double-immunostaining of the sections with an anti-CD133 antibody and an anti-EF5 antibody revealed that CD133 expression was upregulated in some, but not all, hypoxic (EF5-positive) cells compared to normoxic (EF5-negative) cells (Figure 6E).

HIF-1 induces changes in SP-C and stem cell-related gene expression in A549 cells

To investigate whether the hypoxia-induced changes in the expression of *SP-C*, *CD133*, *OCT4*, and *MSI1* were mediated by HIFs, we treated A549 cells with DFO, a hypoxia mimetic. DFO treatment induced a decrease in the expression of *SP-C* and an increase in the expression of *CD133*, *OCT4*, and *MSI1* (Figure 7A, B). Cobalt chloride, which is another hypoxia mimetic, induced similar changes in A549 cells (Figure 7C, D). To obtain more direct evidence of the importance of HIFs in hypoxia-induced changes, we transfected A549 cells with plasmids constitutively expressing CA-HIF-1 α (pcDNA3.1/HIF-1 α ^{P402A/P564A}). CA-HIF-1 α overexpression resulted in a decrease in *SP-C* expression and an increase in *CD133* and *MSI1* expression (Figure 7E, F). The expression of *OCT4* did not change, which was consistent with the report that *OCT4* is a direct target of HIF-2 α but not HIF-

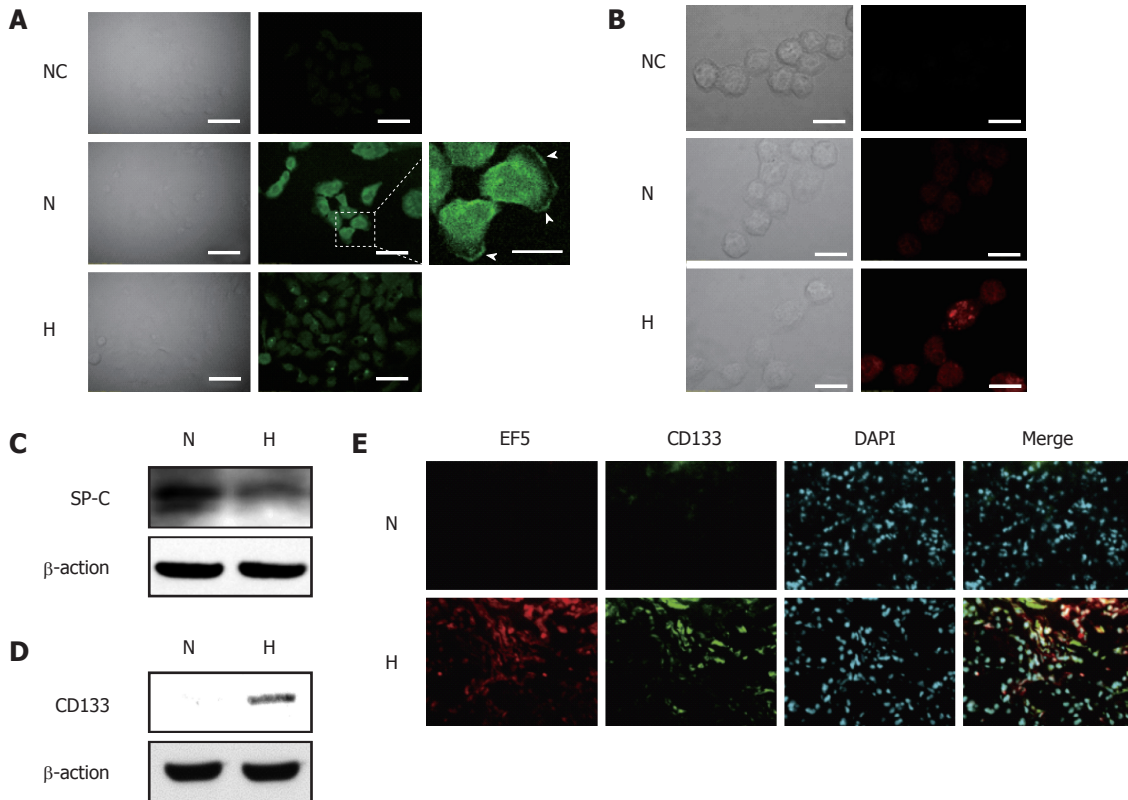


Figure 6 Expression of SP-C and CD133 proteins in hypoxic A549 cells *in vitro* and *in vivo*. A549 cells were cultured under normoxic (N) or hypoxic (H) conditions for 5 d; A: Immunostaining for SP-C, arrowheads indicate the localization of SP-C proteins at cell membranes, NC indicates negative control (normal rabbit serum), Scale bars = 50 μ m (white bars) and 20 μ m (yellow bars); B: Immunostaining for CD133. Formaldehyde-fixed, nonpermeabilized cells were immunostained with phycoerythrin-conjugated monoclonal anti-CD133 antibody, NC indicates negative control, scale bars = 20 μ m; C: Western blot analysis of SP-C protein expression; D: Western blot analysis of CD133 protein expression, C and D total cell lysates were subjected to immunoblot analysis for SP-C and CD133, β -Actin was used as a loading control; E: CD133 expression in hypoxic cells in A549 subcutaneous tumors, tissue sections were double-stained with an anti-CD133 antibody (green) and an anti-EF5 antibody (red). Nuclei were stained with DAPI. Upper panels represent the EF-5-negative (normoxic) area and bottom panels represent the EF-5-positive (hypoxic) area, scale bars = 100 μ m.

1 α ^[9]. These results indicate that HIF-1 induces changes in the expression of SP-C, CD133, and MSI1.

To gain some insight into the mechanism underlying the HIF-mediated suppression of *SP-C* expression in A549 cells, we examined the effects of trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, and 5-azacytidine, an inhibitor of DNA methyltransferase. We showed that TSA but not 5-azacytidine efficiently diminished the suppressive effect of hypoxia on *SP-C* expression (Figure 7G, H), thereby suggesting that HDAC is involved in hypoxia-induced gene expression changes in A549 cells.

TX-402 inhibits the expression of HIF-1 α and HIF-2 α and restores the hypoxia-induced gene expression changes in A549 cells

TX-402 has been shown to inhibit the expression of HIF-1 α protein^[26-28], but its effect on HIF-2 α protein expression remains to be tested. Thus we examined whether TX-402 inhibited the expression of HIF-1 α and HIF-2 α in A549 cells under hypoxic conditions. HIF-1 α and HIF-2 α accumulated after incubating the cells under hypoxic conditions for 9 h. Treatment with 20 μ mol/L TX-402 significantly suppressed the accumulation of both sub-

units, but the levels of HIF-1 α and HIF-2 α mRNA were unaffected (Figure 8A, B).

The proliferation of A549 cells was inhibited by TX-402 (IC₅₀ value of approximately 20 μ mol/L) without any discernible cell killing under hypoxic conditions over a culture period of 3 d (Figure 8C). We then treated the cells under hypoxic conditions for 3 d in the presence of 20 μ mol/L TX-402 and examined the expression of *SP-C*, *CD133*, *OCT4*, and *MSI1*. We found that TX-402 restored the expression levels of *SP-C*, *CD133*, *OCT4*, and *MSI1* in hypoxia to the normoxic levels (Figure 8D). These results were also corroborated by an immunofluorescence study (Figure 8E). We detected nuclear OCT4 and MSI1 using nuclear extracts and found that TX-402 restored the levels of OCT4 and MSI1 in hypoxia to their normoxic levels (Figure 8F).

TX-402 abrogates the hypoxia-induced lung-colonizing potential of A549 cells

We examined the lung-colonizing potential of A549 cells that were cultured under hypoxic conditions after injecting them into the tail veins of nude mice. As evidenced by macroscopic and histological observations, hypoxic A549 cells formed a larger number of metastatic foci in

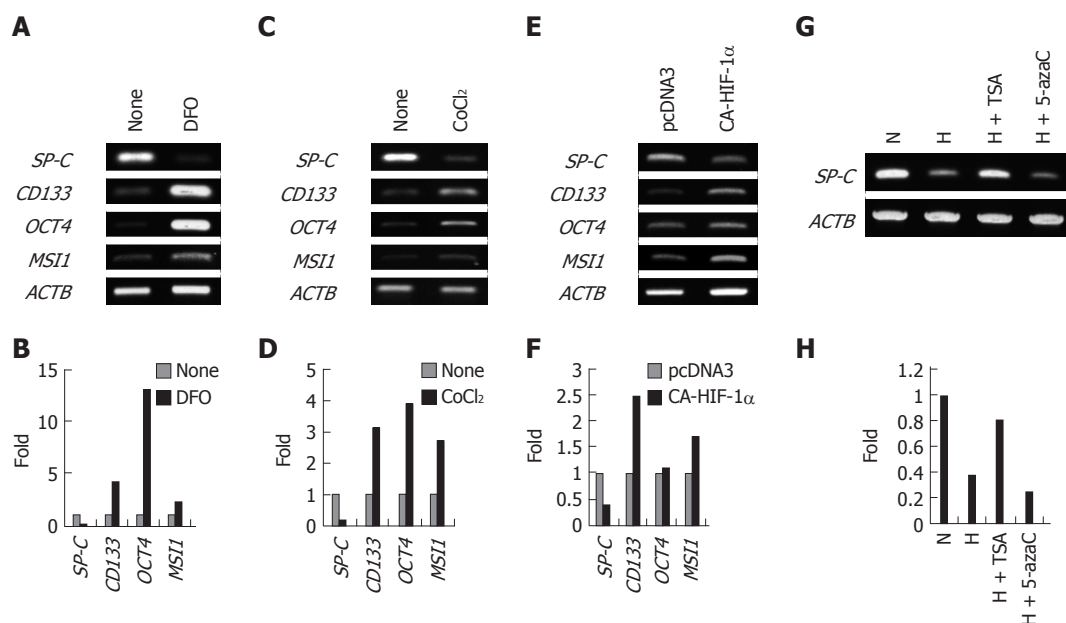


Figure 7 Hypoxia-inducible factor mediates the expression of differentiation- and stem cell-related genes in A549 cells. A and B: Effect of DFO, the cells were cultured with or without 100 $\mu\text{mol/L}$ DFO for 3 d; C and D: Effect of CoCl₂, the cells were cultured in the presence or absence of 200 $\mu\text{mol/L}$ CoCl₂ for 3 d; E and F: Effect of the ectopic expression of HIF-1 $\alpha^{\text{P402A/P564A}}$, the cells were transfected with pcDNA3.1 or pcDNA3.1/HIF-1 $\alpha^{\text{P402A/P564A}}$ and allowed to grow for 2 d; G and H: Effects of TSA and 5-azacytidine on the hypoxia-induced repression of *SP-C* expression, the cells were cultured under normoxic (N) or hypoxic (H) conditions in the presence or absence of TSA (300 nmol/L) or 5-azacytidine (5-azaC) (4 $\mu\text{mol/L}$) for 3 d. B, D, F, and H: The expression level of each gene was normalized to that of β -Actin (*ACTB*), data are shown as fold-change relative to normoxia (normoxia values set to equal 1).

the lungs than did normoxic A549 cells (Figure 9A, B). The lung-colonizing ability of hypoxic A549 cells was abolished by TX-402 (Figure 9C, D). To exclude the possibility that this suppressing effect of TX-402 was due to impaired colony-forming ability and enhanced senescence induction, we examined the effect of TX-402 on the colony-forming ability of A549 cells. Although the colony-forming ability of the cells that were treated with 20 $\mu\text{mol/L}$ TX-402 under hypoxic conditions for 3 d was slightly but significantly inhibited (Figure 9E, F), the inhibitory effect of TX-402 on the lung-colonizing ability was more profound.

DISCUSSION

Here we show that A549 cells primarily express *SP-C*, which indicates that most of these cells are in the AT2 cell lineage. These cells also express the *CD133*, *OCT4*, *MSI1*, *SOX2*, and *GLDC* stem cell markers, suggesting that A549 cells comprise subpopulations of stem and progenitor cells. The exposure of A549 cells to hypoxia had the following effects without any sign of cell death: suppression of the expression of *SP-C*; upregulation of the expression of *CD133*, *OCT4* and *MSI1*; and slight upregulation of the expression of *NANOG*. A549 cells have been shown to form iPSC-like colonies when introduced to Oct4, Sox2, Nanog and Lin28 together with a non-degradable form of HIFs^[35]. Our results were in agreement with these data and further provide important information that tumor hypoxia itself can render CSC-like NSCLC cells.

Recent studies have shown that CD133-positive tumor cells exhibit higher tumorigenicity, clonogenicity, and metastatic ability than CD133-negative cells in different types of cancers, including primary non-small cell and small cell lung cancers^[36-41]. Therefore, the hypoxia-induced expression of CD133 might partly contribute to the CSC-like phenotype of hypoxic A549 cells. OCT4 is overexpressed in bladder cancer, and ectopic expression of OCT4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues^[42,43]. Furthermore, recent reports have demonstrated that OCT4 expression is associated with the differentiation state of various cancer cells^[44,45], and that it is essential for the successful reprogramming of somatic cells to induced pluripotent stem cells^[46]. NANOG functions to maintain the pluripotency and co-expression of OCT4, and it is necessary for inducing the CSC-like properties of A549 cells^[47]. MSI1 is an RNA-binding protein that is linked to asymmetric cell division^[48]. Based on these reports, our results suggest that hypoxia induces the dedifferentiation of A549 cells. However, although we found that A549 cells express *GLDC*, its expression was not influenced by hypoxia. Because murine BASCs are SP-C/CC10 double-positive^[17,18,48] and the recently identified putative mouse lung stem/progenitor cell population is CC10-positive/SP-C-negative^[49], the properties of hypoxic A549 cells do not closely conform to those known to be characteristic of stem/progenitor cells.

The mechanism underlying the hypoxia-induced suppression of *SP-C* involves HIF-1 α at least in part, because the overexpression of CA-HIF-1 α suppressed *SP-C* ex-

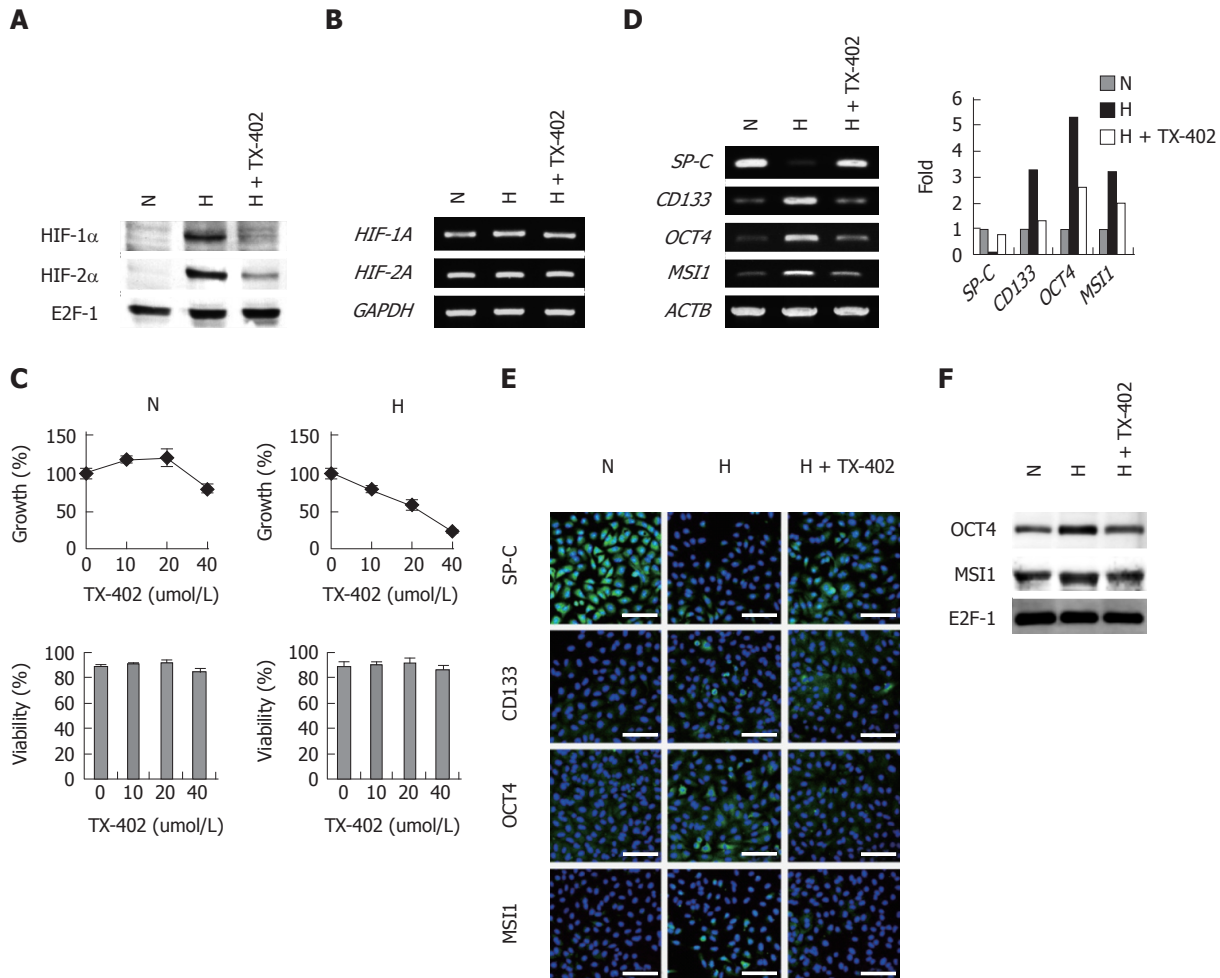


Figure 8 Effects of TX-402 on hypoxia-inducible factor- α expression, cell proliferation, viability and gene expression levels of A549 cells. The cells were cultured under normoxic (N) or hypoxic (H) conditions in the presence or absence of 20 μ mol/L TX-402 for 3 d; **A**: Expression of HIF- α subunits, Nuclear extracts and total RNA were subjected to Western blot; **B**: Expression of HIF- α subunits, Nuclear extracts and total RNA were subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis; **C**: Cell growth and viability, bars, SD ($n = 3$); **D**: RT-PCR analysis of the expression of SP-C, CD133, OCT4 and MSI1 Mma, the expression level of each gene was normalized to that of β -Actin (ACTB), data are shown as fold-change relative to normoxia (normoxia values set to equal 1); **E**: Immunofluorescence analysis of the expression of SP-C, CD133, OCT4 and MSI1 protein, Nuclei were stained with DAPI, and the merged images are shown, Perinuclear and nuclear localization of OCT4 and MSI1 was observed, Scale bars = 50 μ m; **F**: Western blot analysis of the nuclear localization of OCT4 and MSI1, Nuclear extracts of the cells were subjected to the analysis, E2F-1 was used as a loading control.

pression. Because TSA reversed the suppressive effect of hypoxia, HDAC is also likely to be involved in the mechanism. Alternatively, because HIF-1 α is post-translationally modified by acetylation of lysine residues within the N terminus leading to the stabilization of the protein^[50], it is possible that TSA reduces the level of HIF-1 α by directly acting at the protein level, which in turn restores hypoxia-induced SP-C repression to normal levels. In contrast, the upregulation of CD133, OCT4, and MSI1 in hypoxic A549 cells is undoubtedly mediated by HIFs because treatment with the hypoxia mimetics, namely DFO and CoCl₂ (both of which stabilize HIF- α subunits) upregulated all of them. Furthermore, we showed that CA-HIF-1 α overexpression up-regulated CD133 and MSI1 but not OCT4, which is an HIF-2 α -specific target gene^[11].

It is thought that CSCs display self-renewing ability, a high capacity for tumor initiation, and a high metastatic potential. We used sphere formation and lung-colonizing

assays to examine whether hypoxic A549 cells also have these functional phenotypes. The lung-colonizing assay can examine the tumor-initiating capacity and growth of a single cell or a small mass of cells in orthotopic sites that mimic the *in vivo* niche conditions of CSCs in NSCLC. In the present study, we showed that hypoxic A549 cells formed larger spheres and more lung nodules after intravenous implantation compared to normoxic A549 cells. This result suggests that hypoxic A549 cells have high self-renewing activity, tumor-initiating capability, and/or metastatic ability. Thus, based on the gene expression data and functional studies, we conclude that hypoxia is able to induce subpopulations of A549 cells with CSC-like phenotypes.

To determine whether our findings could be generalized to other cell types, we also investigated the effect of hypoxia on QG56 cells, and we observed that the expression of the CC10 and AQP5 differentiation marker

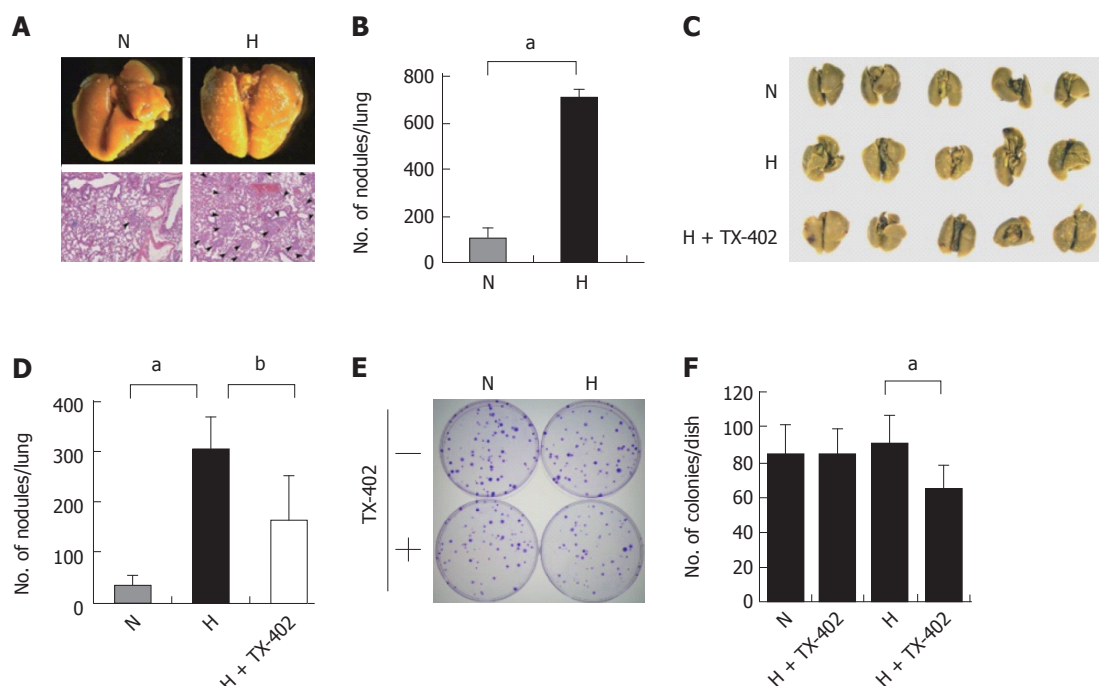


Figure 9 Effect of TX-402 on the lung-colonizing capability of A549 cells cultured under hypoxic conditions. A, B: Macroscopic and histological observations of the lungs. A549 cells cultured under normoxic (N) or hypoxic (H) conditions for 5 d were injected into the tail vein of Balb/c nude mice ($n = 5$), the lungs were processed for macroscopic and histological (hematoxylin and eosin staining) observations, and the number of metastatic foci was counted, data are presented as the mean \pm SE, $^bP < 0.01$; C, D: Effect of TX-402 on the hypoxia-induced lung-colonizing ability of A549 cells. A549 cells cultured under normoxic (N) or hypoxic (H) conditions with vehicle or 20 $\mu\text{mol/L}$ TX-402 for 3 d were injected into the tail vein of BALB/c nude mice ($n = 5$). The lungs were fixed, and the number of nodules per lung was then counted. Data are presented as the mean \pm SE, $^bP < 0.01$ and $^aP < 0.05$; E, F: Colony-forming ability of TX-402-treated A549 cells, A549 cells (100 cells/dish) cultured under normoxic (N) or hypoxic (H) conditions in the presence of solvent (DMSO) or 20 $\mu\text{mol/L}$ TX-402 for 3 d were seeded and cultured for an additional 14 d, the colonies were stained with crystal violet, data are presented as the mean \pm SD ($n = 6$), $^bP < 0.01$.

genes was markedly suppressed under hypoxic conditions. Inversely, *CD133*, *OCT4*, and *MSI1* were up-regulated in hypoxic QG56 cells, and hypoxia weakly enhanced the sphere-forming capacity of QG56 cells. These results suggest that hypoxia also induces dedifferentiation of the cells.

An important finding of our study is that TX-402 blocked hypoxia-induced changes in the expression of the stem cell-related genes and an increase in the lung-colonizing ability of A549 cells, which most likely occurred *via* inhibition of the expression of HIF- α subunits. Although we observed a slight reduction in the survival of cells that were treated with TX-402 under hypoxic conditions, TX-402 was not cytotoxic at the concentration used. Therefore, it is likely that in addition to its growth-inhibitory effect in mild hypoxia, TX-402 inhibited the lung-colonizing ability of A549 cells by repressing cell dedifferentiation. Further studies using other NSCLC cell lines or primary patient samples are required to generalize the effects of TX-402 on dedifferentiation and lung-colonizing ability.

In conclusion, our results suggest that hypoxia induces the dedifferentiation of NSCLC cells into CSC-like cells with high metastatic potential and that HIF inhibitors, such as TX-402, may prevent this process. Recent studies have demonstrated that hypoxia in tumors can be induced by the administration of antiangiogenic agents, such as bevacizumab and VEGF receptor tyrosine kinase inhibitors, and that intermittent use of these drugs ac-

celerates tumor growth and metastasis presumably by increasing the CSC population^[6-8]. Therefore, combination therapy where antiangiogenic agents are combined with HIF-targeting drugs could be effective in improving patient outcomes. Thus, further studies on HIF-targeting drugs are warranted to determine their full potential in the treatment of disease.

ACKNOWLEDGMENTS

We thank Dr. N. Koshikawa for helpful discussions.

COMMENTS

Background

Hypoxia influences many aspects of cancer cell biology, including neoangiogenesis, energy metabolism, cell survival, radiosensitivity, chemosensitivity, differentiation and invasion/metastasis, via hypoxia-inducible factors (HIFs). Hypoxia also induces the dedifferentiation of some tumor cells, rendering them more cancer stem cell (CSC)-like and metastatic.

Research frontiers

The effects of hypoxia on the dedifferentiation and maintenance of CSC phenotypes of non-small cell lung cancer (NSCLC) is largely unknown. Furthermore, it remains to be examined whether HIF inhibitors can suppress the hypoxia-induced process.

Innovations and breakthroughs

In this study, the authors demonstrate that hypoxia induces the dedifferentiation of NSCLC and that TX-402, a small-molecule inhibitor of HIF-1 α and HIF-2 α expression, can suppress the hypoxia-induced process and, importantly, metastatic ability.

Applications

By understanding how tumor hypoxia induces the dedifferentiation of NSCLC, this study could represent a future strategy for therapeutic intervention in the treatment of patients with NSCLC.

Terminology

Tumor hypoxia is generated in most solid tumors because of a shortage in oxygen supply. HIF is a transcription factor that is composed of HIF- α and HIF- β subunits, and it plays a central role in hypoxia-induced biological processes. CSCs are defined as those cells within a tumor that can self-renew, drive tumorigenesis, exhibit chemoresistance, exhibit radio-resistance, and have high metastatic potential.

Peer review

The authors describe an inhibitor of the HIF- α subunit expression that suppresses the hypoxia-induced dedifferentiation of human NSCLC cells into CSC-like cells. This article was highly evaluated because the authors examined the hypoxic effect on NSCLC cells in detail.

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P- Reviewers: Javier C, Kanno H, Scatena R

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