Identification of mutations in cancer predisposition genes in radiosensitive and radioresistant patients with nasopharyngeal carcinoma.

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Abstract

The objective of the present study was to determine whether the alterations of cancer predisposition genes were different between radiosensitive and radioresistant patients with Nasopharyngeal Carcinoma (NPC). A total of 21 patients with nasopharyngeal carcinoma were included in this study. All patients were treated with standardized radiotherapy. Sixteen of the tumors were clinically radiosensitive and 5 were radioresistant. Genomic DNA, extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tumor specimens obtained prior to treatment, was subjected to amplicon-based Next-Generation Sequencing (NGS) with primer sets targeting 50 critical human tumor suppressor genes and oncogenes. We identified 18 nonsynonymous mutations, 1 nonframeshift deletion and 1 frameshift mutation distributed across 15 genes, including 11 mutations have been reported in COSMIC (the Catalogue of Somatic Mutations in Cancer) or dbSNP database (database of single nucleotide polymorphisms), and 9 novel mutations. Most of these mutations have not been reported in NPC. More importantly, 5 radiosensitive-specific mutations targeting *AKT1, PIK3CA, MET, TP53* and *STK11* were observed, suggesting the genetic alterations of *PI3K/AKT* and *p53* pathways were involved in the response to radiotherapy. Collectively, genetic mutations may differentiate tumor radiosensitivity and radioresistance, although validation of such mutations using a large sample size cohort is necessary before a solid conclusion can be reached.

Introduction

Nasopharyngeal Carcinoma (NPC) is extremely common in southern China, particularly in Guangdong, accounting for 18% of all cancers in China [1]. Epstein-Barr virus infection, heredity and environmental influences are major causes of NPC [2-4]. Most NPC patients are sensitive to radiotherapy, and combined radiochemotherapy may improve the survival of these patients [5,6]. However, radioresistance remains a serious problem affecting the treatment of NPC in many cases. If patients who tend to be resistant to radiotherapy can be identified before treatment and receive a higher dose of therapy without increased the rate of complications, the outcome of radiotherapy or combined radiochemotherapy may be significantly improved.

Microarrays and proteomics have been applied to identify radioresistance associated genes or proteins in multiple types of cancer, including NPC [7-15]. A number of studies have focused on the association between the radiosensitivity of various cancers and genetic changes of oncogenes and tumor suppressor genes, such as *p53* [16-18], *ATM* (19), Epidermal Growth Factor Receptor (EGFR) [20,21]. Although mutations of RB2/p130 [22], *p53* [23,24], *PIK3CA* [25-27] and *EGFR* [28] have been investigated in NPC, little is known about radioresistance-associated mutations in NPC.

With the advances in Next-Generation Sequencing (NGS) techniques, a large number of mutations have been identified in cancers [29]. Multiplex amplicon-based NGS, sequencing only the Regions of Interest (ROI), has several advantages: requires relatively small amounts of DNA, is relatively low cost and is compatible with Formalin-Fixed Paraffin-Embedded (FFPE)-DNA [30]. In the current study, genomic DNA was extracted from FFPE tissue samples from radiosensitive and radioresistant patients with NPC, and then the hotspot regions of 50 critical human tumor suppressor genes and oncogenes were amplified. Amplicon-based NGS was carried out to

investigate any potential correlation of mutational hot spots with radiosensitivity.

Materials and Methods

Collection of NPC specimens

A total of 21 Formalin-Fixed Paraffin-Embedded (FFPE) samples were taken form Shandong Provincial Hospital Affiliated to Shandong University, China after approval by the local ethics committee. All patients were given written informed content. All patients were treated with two cycles of radiotherapy (50-70 Gy total dose) after cisplatin-docetaxel induction chemotherapy. Four response categories are proposed: complete response (CR, which means the disappearance of all detectable tumor), partial response (PR, which is defined as more than 50% decrease in tumor size), stable disease (SD, which means tumor remains the same size), and Progressive Disease (PD). Radiosensitive patients are those reached CR 2 to 4 w after completion of radiotherapy, and radioresistant patients are clarified as those of PR or SD or even with PD 2 to 4 w after completion of radiotherapy. FFPE specimens were obtained prior to treatment, and 16 patients were found radiosensitive (Samples 1-16) and 5 patients (Samples 17-21) were found radioresistant during follow-up.

DNA isolation and amplicon library preparation

Genomic DNA was extracted separately from FFPE samples using QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The concentrations of isolated FFPE-DNA samples were determined on a Qubit 2.0 Fluorometer (Life Technologies).

The amplicon library was prepared using DNA Seq Library Preparation Kit for Amplicon Sequencing-Illumina Compatible (Gnomegen, San Diego, CA), which contains 207 primer pairs and targets 50 proto-oncogenes and tumor suppressor genes (Table 1). Target sequences were amplified with 10 ng FEPE-DNA. Subsequently, end repair, barcode ligation and a simple PCR amplification were performed following the manufacturer's instructions with several purification steps (Gnomegen). The barcode sequences were unique for each sample. Size selection was then performed to obtain clean library products. Following Quality Control, 21 purified and quantified libraries were pooled to form a library product.

Illumina sequencing and sequence assembly

Sequencing was performed on an Illumina Hiseq2000 sequencing platform (Illumina, San Diego, CA) with 150 bp pair reads. After sequencing, the raw data were separately trimmed to remove 5'- and 3'- adapters and low quality regions (<Q20). After discarding reads shorter than 50 bp, reads were mapped against a reference genome using Burrows-Wheeler Alignment tool (BWA). Variances were identified by VarScan software (http://varscan.sourceforge.net) with the following parameters: minimum base quality of 20, minimum coverage of 30X, minimum variation frequency of 3%, no strand bias, and a P value of less than 0.05.

Results

Sequencing data

We applied NGS to investigate mutations of 50 proto-oncogenes and tumor suppressor genes on 5 radioresistant (Samples 17-21) and 16 radiosensitive (Samples 1-16) patients. We generated 1.3-2.4 million sequencing reads per sample (Q20>96% and Q30>93%). As shown in Table 2, the align rate was >98%, on target rate was >93%, the average read depth (the number of times a nucleotide is sequenced) per sample was 5000X, and coverage uniformity was >90%. Thus, the sequencing data allowed the detection of variances.

Frequency and distribution of variances

After data processing, 67 variances were detected in the 21 samples (Table S1). Then, we excluded 27 variances within Untranslated Region (UTR) and intron, as well as 20 synonymous mutations (no amino acid change occurred), and obtained 20 distinct variants (18 nonsynonymous, 1 nonframeshift deletion and 1 frameshift) of 15 genes (*PTEN, HRAS, AKT1, IDH2, CDH1, TP53, SMAD4, STK11, PIK3CA, FGFR3, KDR, APC, MET, FGFR1* and *NOTCH1*) (Table 3). Of the 20 distinct variants, 10 were found in COSMIC database (the Catalogue of Somatic Mutations in Cancer) [31] and 8 variants were found in dbSNP database (database of single nucleotide polymorphisms) [32]. Nine novel variants, which were not found in COSMIC database or dbSNP, were then subjected to Polyphen2 (http://genetics.bwh.harvard.edu/pph2/). The results showed that 8 novel variants were predicted to be "probably damaging" or "possibly damaging".

Screening for radioresistance-associated variances

By comparing the 20 variances occurred in radiosensitive and radioresistant samples, we found that 5 variants occurred only in radiosensitive samples, but not in radioresistant samples. These 5 variants may be associated with radioresistance (Table 3). No obvious difference was observed in other 15 variants between the two groups of samples.

***Table 1.** The panel of 50 proto-oncogenes and tumor suppressor genes.*

ABL1	AKT1	ALK	APC	ATM	BRAF	CDH1	CDKN2A	CSF1R	EZH2
CTNNB1	EGFR	ERBB2	ERBB4	FBXW7	FGFR1	FGFR2	FGFR3	FLT3	GNA11

GNAS	GNAQ	HNF1A	HRAS	IDH1	IDH2	JAK2	JAK3	KDR	KIT
KRAS	MET	MLH1	MPL	NOTCH1	NPM1	NRAS	PDGFRA	PIK3CA	PTEN
PTPN11	RB1	RET	SMAD4	SMARCB1	SMO	SRC	STK11	TP53	VHL

Table 2. *Quality control of sequencing data.*

Sample	Aligned reads (M)	Align rate	On target reads (M)	On target rate	Mean depth (1000x)	Specificity	Coverage uniformity
1	1.929	99.34%	1.889	97.91%	8.847	97.13%	91.41%
2	1.678	99.31%	1.637	97.54%	7.661	96.79%	90.55%
3	1.322	99.40%	1.301	98.47%	6.128	97.84%	89.10%
4	1.568	99.28%	1.528	97.44%	6.994	96.40%	90.85%
5	1.556	99.41%	1.504	96.67%	7.149	96.12%	78.11%
6	2.423	99.30%	2.362	97.48%	11.123	96.67%	90.52%
7	1.568	99.16%	1.514	96.59%	7.105	95.75%	81.89%
8	1.966	99.11%	1.894	96.38%	8.898	95.50%	82.63%
9	1.71	99.27%	1.678	98.13%	7.893	97.41%	93.80%
10	1.67	98.78%	1.636	97.98%	7.605	96.69%	87.29%
11	1.68	99.27%	1.645	97.92%	7.762	97.15%	86.70%
12	2.012	99.24%	1.967	97.74%	9.192	96.97%	85.00%
13	1.674	98.69%	1.646	98.34%	7.574	96.71%	89.75%
14	2.273	99.95%	2.249	98.91%	3.076	89.14%	74.06%
15	1.962	99.92%	1.936	98.67%	3.239	86.72%	76.50%
16	1.844	99.97%	1.808	98.08%	3.229	88.53%	77.91%
17	2.017	99.98%	1.978	98.04%	3.333	88.10%	78.40%
18	1.602	99.97%	1.573	98.19%	3.051	88.14%	79.51%
19	1.925	99.98%	1.902	98.82%	3.187	85.83%	75.21%
20	1.971	99.11%	1.918	97.30%	9.112	96.43%	93.22%
21	1.854	99.36%	1.73	93.29%	8.248	92.60%	87.56%

Table 3. *Identified distinct variants.*

	Gene	Accession	Chromosome	Mutations	Cosmic	dbSNP	Polyphen-2
Radiosensitive	AKT1	NM_001014431	chr14	E17K	COSM33765	rs121434592	
	TP53	NM_001126118	chr17	S51fs	COSM131026, COSM131025, COSM131024		
	STK11	NM_000455	chr19	F354L	COSM21360	rs59912467	
	PIK3CA	NM_006218	chr3	R108H	COSM27497, COSM342716		
	MET	NM_000245	chr7	N375S	COSM710	rs33917957	
Others	PTEN	NM_000314	chr10	Q110E	no entry		0.001 (benign)
	HRAS	NM_001130442	chr11	G13D	COSM490	rs104894226	
	IDH2	NM_001290114	chr15	P32H	no entry		0.998 (probably)

CDH1	NM_004360	chr16	P372H	no entry		0.999 (probably)
TP53	NM_001126118	chr17	P33R	COSM250061	rs1042522	
SMAD4	NM_005359	chr18	D424N	no entry		0.575 (possibly)
PIK3CA	NM_006218	chr3	Y68C	no entry		1 (probably)
PIK3CA	NM_006218	chr3	E545A	COSM297145, COSM12458	rs121913274	
PIK3CA	NM_006218	chr3	L719V	no entry		0.849 (possibly)
FGFR3	NM_000142	chr4	V266M	COSM29434		
KDR	NM_002253	chr4	Q472H	COSM149673	rs1870377	
APC	NM_000038	chr5	A1582T	no entry		1 (probably)
APC	NM_000038	chr5	A1582P	no entry		1 (probably)
FGFR1	NM_001174066	chr8	43_44del	no entry	rs138489552	
NOTCH1	NM_017617	chr9	E1567D	no entry		0.549 (possibly)

Discussion

Radioresistance remains a major obstacle to the treatment of NPC. Genetic changes have been reported to be associated with the radiosensitivity of various cancers. Identification of NPC radioresistance-associated mutations will help finding biomarkers to predict the response to radiotherapy and explaining the molecular mechanisms of NPC radioresistance. As far as we known, this study represents the first sequencing study of multiple oncogenes and tumor suppresser genes in radiosensitive and radiotolerant NPC patients.

In this study, NPC samples were tested with a 50-gene hotspot panel, of which, mutations in *PIK3CA, NRAS, KIT, PDGFRA, ABL, HRAS, BRAF* and *EGFR* [24,26-28,33,34] have been reported in NPC. Here, we identified 18 nonsynonymous mutations, 1 nonframeshift deletion and 1 frameshift mutation distributed across 15 oncogenes and tumor suppresser genes, of which 8 mutations were previously observed in dbSNP and 10 mutations, were already known Cancer-Associated Mutations (COSMIC). By analyzing with Polyphen-2 Software, 8 of the 9 novel mutations were predicted to be damaging. These results suggested that we have identified novel mutations which may be associated with tumorigenesis. Alterations in most of the 15 genes have not been reported in *NPC*, except *PI3CA* and *TP53*. Our study also provided novel information regarding gene alterations during *NPC* tumorigenesis.

The PI3K/AKT pathway is known to regulating radioresistance in cancer cells [35-37] and experimental animals [38]. *p53* is a well-known regulator of radiosensitivity in tumor cells [39,40]. By comparing radioresistant and radiosensitive samples, 5 radiosensitive-specific mutations, *AKT1* (COSM33765), *PIK3CA* (COSM27497, COSM342716), *MET* (COSM710), *TP53* (COSM131026, COSM131025 and COSM131024) and *STK11* (COSM21360), were identified, suggesting the genetic alterations of PI3K/AKT and *p53* pathways were involved in the response to radiotherapy. However, these mutations should be validated by large sample size cohort. *In vitro* and *in vivo* experiments with these mutations may further elucidate the underlying mechanisms.

In summary, the present study has identified several candidate variants in NPC patients that may be used to predict increased radiosensitivity although the sample size was relatively small. Our findings reported may be used to distinguish radiosensitive and radioresistant patients before treatment, thus driving personalized therapeutic strategies and improving the therapy outcome.

Acknowledgments

Competing Interests

No conflict of interest to declare.

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