RESEARCH COMMUNICATION

Genome-wide Evidence of *XPC* Alteration in Laryngeal Squamous Cell Carcinomas

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Abstract

Background: There are discrepancies in data of genome-wide microarray-based comparative genomic hybridization (CGH) in squamous cell carcinoma of the head and neck (SCCHN). Variations in ethnic background, life style, presence of chemotherapy, and regions of SCCHN may explain the results. Methods: Considering the variations, we performed CGH in Korean laryngeal SCC (LSCC) tissues (N=16). Results: We found gains of amplification at 7q35 and 8q24 and losses at 1p21, 2q21, 17q12, and 3p22-26 in 40-50 % of the cases. The regions of losses at 3p22-26 contain no known tumor suppressor gene. However, *XPC*, a key gene in DNA repair pathway, is identified at 3p22-25. Conclusion: Our finding strongly suggests that chromosome 3p22-p26 region harbor critical gene(s) including *XPC* associated with risk for LSCC.

Keywords: Head and neck SCC - comparative genomic hybridization - XPC - laryngeal

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Introduction

Most of head and neck cancer occur in squamous cells of the upper respiratory and digestive tracts. These cells are susceptible to tobacco, alcohol, stimulation of various pollutants, and virus infection and altered into cancer cells. That is, the squamous cell carcinoma of the head and neck (SCCHN) is a typical cancer following carcinogenesis by mutations. Several genetic or phenotypical variations in carcinogen metabolic enzymes and DNA repair genes, e.g. xeroderma pigmentosum group C (*XPC*) (Ho et al., 2007), have been suggested as risks of SCCHN. However, most of these studies were not obtained from genome wide analyses. Thus, advanced and integrated genome wide analyses have been asked to overcome overestimated results.

XPC, a key gene of DNA nucleotide excision repair pathway, has been investigated in many studies of SCCHN susceptibility and showed potential roles in etiology of SCCHN: The majority of these studies have focused on genetic polymorphisms in *XPC*. Previous studies reported that single nucleotide polymprhisms (SNPs) of *XPC* are significantly associated with risk for SCCHN (Francisco et al., 2008), although some of these data were not confirmed in laryngeal SCC (LSCC) (Abbasi et al., 2009). In addition, we previously reported loss of *XPC* expression rather than the *XPC*-PAT genetic polymorphism as a risk for SCCHN in Korean population (Yang et al., 2005).

However, there are some limitations of a global or non-invasive gene, such as *XPC*, to be a critical etiology for SCCHN, because multiple genetic factors and steps are involved in SCCHN initiation and progression. Thus, full genome wide studies are a desirable methodology to confirm or clarify real etiologies of SCCHN. Due to the technical development for molecular biology, integrated genome wide approaches were available to investigate cancer etiology. For example, chromosomal comparative genomic hybridization (CGH) is a well-established technique to evaluate the entire tumor genome. Some researchers have evaluated the entire genome using CGH to clarify genetic alterations in head and neck cancer: In a review, the most frequent cytogenetic alterations in SCCHN were gains on 3q, 5p, 7p, 8q, 9q, 20q, and 11q13, and losses on 3p, 5q, 8p, 9p, 13q, 18q, and 21q1 (Gollin et al., 2001; Akervall, 2005). From comparison of regions in SCCHN, Patmore reported that 50% of laryngeal, oropharyngeal and hypopharyngeal SCC showed chromosomal aberration with +3q, -3p, and +8q (Patmore et al., 2005). In addition, 7 and 8 chromosomal aberration are recently emphasized to be influenced by formaldehyde from occupational or environmental exposure including indoor air pollution (NTP, 2010).

However, there are some discrepancies between those whole genome studies in SCCHN: Juhasz et al. reported alteration at +3q and +8q in untreated laryngeal tumor from Hungarian (Juhasz et al., 2005). On the other hand, a Polish study showed gains on 1p, 9q, and 16q and losses on 13q (Schlade-Bartusiak et al., 2005). In addition, Singh et al. reported that gains at +1p and +3q were more common in patients with a history of tobacco/alcohol exposure,

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however, half of the whole American subjects in their study were treated adjuvant radiation (Singh et al., 2002). Recently, Keser et al. reported gains at 17p, 3p, 4p, 5p, 6q, 8p, 9p, 14q, and 18p and Xq and losses at 15q and 22q in Turkey LSCC (Keser et al., 2008). Thus, variations in ethnic background, life style, presence of chemotherapy, and regions of SCCHN can explain the discrepancies of the CGH results. Most genome wide CGH studies were performed in Caucasians and Africans (Natasya et al., 2010). Considering these factors, we performed CGH analyses to characterize genome wide features in Korean LSCC, and to clarify the roles of *XPC* on etiology of SCCHN.

Materials and Methods

Patients and tumor specimens

The subjects were recruited in the Department of Otolaryngology, Hanyang University (Seoul, Korea) between 2006-2009. To eliminate potential confounding effects from treatment, we only included incident and untreated patients without previous radiation exposure. All patients were histopathologically confirmed laryngeal squamous cell carcinoma (N=16). All subjects (age, 55.6 ± 10.8 yrs) completed a questionnaire to ascertain their environmental and lifestyle characteristics.

We obtained an approval from the Hanyang University Research Ethics Committee and informed consent from the patients. We took tumor tissues, which were identified by an expert of pathology, for CGH analyses from the patients and immediately froze them in liquid nitrogen. For controls for CGH, we used DNA samples of healthy Koreans, which were provided from Macrogen, Inc. (Seoul, Korea).

CGH analyses

The CGH was performed with the standard procedures. First, DNA specimens were extracted from each tissue using PureGene kit (Gentra systems Inc., Minneapolia, MN, USA).

1. Construction of BAC-mediated array CGH microarray: Bacterial artificial chromosomes (BACs) were selected from Macrogen's proprietary BAC library (http://www.macrogen.com). Size selected HindIII digested pooled human DNA and pECBAC1 was used to generate a BAC library. The vectors for this library were transformed and grown in DH10B.

The clones were first selected bioinformatically to give an average genomic coverage of 2 Mb resolution. All of the clones were two end-sequenced and their sequences were blasted and mapped according to their positions on UCSC human genome database (http://www.genome.uscs.edu). Confirmation of locus specificity of the chosen clones was performed by removing multiple loci binding clones by individually examining. These clones were prepared by conventional alkaline lysis method to obtain BAC DNA. The DNA was sonicated to generate about 3kb fragments before mixing with 50% DMSO spotting buffer. Each BAC clones were represented on an array as triplicated spots and each array was represented pre-scanned using Axon scanner for proper spot morphology. The array used in this study consists of 1,440 human BACs, which were spaced approximately 2.3 Mb across the whole genome (MacroArray™ Karyo 1400 from Macrogen, Inc).

2. DNA labeling for array CGH and Array hybridization: For the labeling, hybridization, and imaging, we followed Han et al.' method (Han et al., 2006). Briefly, DNA specimen (500ng) from healthy Korean control (Macrogen-provided reference) or SCCHN patient were labeled using BioPrime ®DNA Labeling System and BioPrime® Array CGH Genomic Labeling System. After labeling, in one tube, Cy3-labeled sample and Cy5-labeled reference were mixed together, and 100 μg of human Cot I DNA (Invitrogen), the DNA was resuspended in a hybridization solution containing 50% formamide, 10% dextran sulfate, 2x SSC, 4% SDS and 200 ug yeast tRNA. Hybridization was performed in slide chambers for 48 hours at 37°C.

3. Array imaging and data analysis: After post hybridization washes, arrays were rinsed, dried with spin, and scanned into two 16-bit TIFF image files using GenePix4200A two-color fluorescent scanner (Axon Instruments, Sunnyvale, CA) and quantitated using GenePix software (Axon Instruments). The log2 transformed fluorescent ratios were calculated from background subtracted median intensity values, and these values were used to normalize according to intensity normalization method. Chromosomal aberrations were categorized as a gain when the normalized log2 transformed fluorescent ratio was higher than 0.25 and as a loss when this ratio was below –0.25.

Statistical analyses

Significant clones were calculated using 2-sample t-test with randomized variance model. The experiments in each of the two groups used for comparison were defined based on the invariant analysis. For testing significant of differences in distribution of categorical variables, cross tables were analyzed with the two-sided Fisher's Exact test. The clones that have $p<0.05$ were considered as significant, unless otherwise specified. We performed multiple testing to further narrow down the significant clones discussed in this paper (Macrogen's MacViewer array CGH software).

Simple regression analyses were performed to analyze association between continuous values, e.g. cigarette-packs per years and copy number alteration on 3p25.1. ANOVA was used to investigate an association between amplification in 3p25.1 and clinical stage. The P-values for all tests were computed by JMP version 4 (SAS Institute, Cary, NC), and $P< 0.05$ was used to identify significant associations.

Results

Characteristics of the subjects

Based on TNM status (AJCC sixth edition), the subjects (15 males, 1 female; 14 smokers, 2 non-smokers;

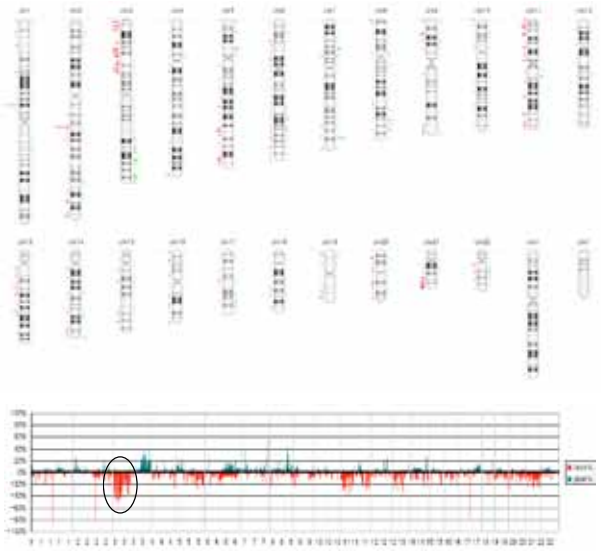

Figure 1. Summary of Genetic Alterations in each Chromosome for 16 SCCHNs. A, Losses of genetic expression are shown to the left and gains to the right of each ideogram. B, Y axis is % of copy number change; circle, the most condensed site of copy number alteration

13 alcohol drinkers, 3 non-drinkers), were classified into stage I (N=4), II (N=1), III (N=2), and IV (N=9).

Altered genes by CGH and status of XPC

Table 1 and Figure 1 summarize the DNA alteration identified in this study. The CGH analysis showed multiple genetic aberrations throughout the genome including (over 40 % changes) -87 % at 1p21.1, -81% at 2q21.1, +56 % at 7q35 , -50 % at 3p25.1, +44 % at 8q24 .12, -44 % at 3p26.3, 3p22.2 and 3p22.3, and -43 % at 3p22.1 and 3p25.2 (Fig. 1): The most notable and novel finding in the present study was losses of 3p 22~26 region (Fig.1). The other common deletions were located at 11q. In detail, we listed the genes on the genetically altered sites from the results of CGH analyses (Table 2): The altered 57 genes were various, and the genes that were at condensed sites, were mainly involved in protein coding. Among the altered genes, *XPC* is located on 3p25.1. In addition, recently emphasized cancer-related genes, e.g. *DLCE1*, *PPARG* and *TIMP4*, were also located on 3p22~25. The most frequently gained regions were localized to chromosomes 3q in 40% of the cases.

Concerning SCCHN progress, we studied copy number changes on 3p25.1. As results, stage 1, 2 or 3 (N=7) showed some reduced copy number on 3p25.1, compared to stage 4 (N=9), however, it was not significant (p=0.47).

Interactions between loss of 3p25.1 and environmental risk factors

To evaluate the interaction between environmental risk factors and common copy number alterations (CNAs), we stratified the patients based on alcohol consumption and smoking. There is no interaction between loss of 3p25.1 and environmental risk factors, such as smoking or alcohol drinking [p= 0.24 or 0.67, respectively, by regression analyses between CNAs on 3p25.1, where *XPC* is located, and cigarette- packs per years or alcohol drinking years].

Table 1. Altered Chromosomes and Genes in the LSCC Patients

Alteration Genes (%) Cytoband	Function with gene ID (NCBI website#)
Gain (56) 7q35	
AKR1B1	Oxidoreductase activity (231)
CNTNAP2	Neurexin family (26047)
FAM115A	Unknown (9747)
OR2A9P	Neuronal response[1] (441295)
OR2A42	Neuronal response[1] (402317)
OR2F1	Neuronal response[1] (26211)
OR2F2	Neuronal response[1] (135948)
OR2Q1P	Neuronal response[1] (346524)
TAS2R60	Bitter taste receptor genes (338398)
TAS2R62P	Taste receptor genes (338399)
TRPV5	Calcium channel activity (194352)
Gain (44) 8q24.12	
MTBP	Protein coding (27085)
NOV	Growth factor activity (18133)
LOC392264	Unknown (392264)
SAMD12	Unknown (320679)
TRPS1	DNA, protein and metal coding (83925)
WISP1	Connective tissue growth factor (CTGF) (8840)
DDEF1	Unknown (50807)
Loss (-87) 1p21.1	
DDX20	Putative RNA helicases (11218)
LOC126987	Unknown (126987)
Loss (-81) 2q21.1	
LOC646674	Unknown
PLEKHB2	Protein coding (55041)
TSN	DNA-coding protein (7247)
Loss (-75) 17q12	
PPP1R1B	Phosphoprotein phosphatase inhibitor (84152)
PSMB3	Peptidase activity (5691)
PSMD3	Enzyme regulator activity (5709)
RAMP2	Receptor (calcitonin) activity modifying (10266)
RASL10B	GTP coding (91608)
RFFL	Ligase, metal and protein coding (117584)
SLC25A39 s	Receptor coding (51629)
Loss (-50) 3p25.1	
LOC728721	Unknown(728721)
LSM3,	Protein coding, RNA coding (27258)
TMEM43	Cellular component (79188)
XPC	DNA repair (7508)
Loss (-44) 3p26.3	
CNTN4/6	Protein coding (152330)(27255)
CRBN	ATP-dependent peptidase activity (51185)
LOC642891	Unknown
Loss (-44) 3p22.3	
CLASP2	Protein coding (23122)
CMTM8	Cytokine activity (152189)
UBP1,	Transcription factor activity (7342)
Loss (-44) 3p22.2	
DLEC1	Negative regulation of cell cycle (9940)
LRRFIP2, 1 2	Wnt receptor signaling pathway (9209)
MYD88	Protein coding (4615)
Loss (-43) 3p25.2	
PPARG	Ligand-dependent nuclear receptor activity (5468)
SYN2	Neurotransmitter secretion (6854)
TIMP4	TIMP metallopeptidase inhibitor 4 (7079)
VGLL4	Regulation of transcription (9686)
Loss (-43) 3p22.1	
MOBP, n	Structural constituent of myelin sheath (4336)
SEC22C, C	Protein transport (9117)
ULK4	ATP coding, nucleotide coding (54986)
ZBTB47	Metal ion coding (92999)
CCK	Protein coding (885)
EPM2AIP1	Protein coding (9852)
KBTBD5	Protein coding (131377)

We listed the genes, which altered to over 40% of gain or loss %: Gain, Normalized log2-Ratio >0.25; Gain %, No. of gained subjects/No. of all subjects x 100 Loss, Normalized log2-Ratio <-0.25; loss %, No. of lost subjects/No. of all subjects x100; [1] that triggers the perception of a smell

In addition, age was not associated with CNAs on 3p25.1, either (p=0.75).

Discussion

Epidemiological studies have shown that environmental factors such as smoking, alcohol consumption, infections, inflammation, and demographic factors, e.g. sex and age, are involved carcinogenesis of laryngeal cancer (Yang et al., 2005). However, individual variations of susceptibility to laryngeal cancer were identified even after adjustment of these risk factors. Therefore, many researchers have investigated genetic individual variations in genes involved in cell cycle, carcinogen metabolism, and DNA repair (Ho et al., 2007). Thus, most studies are utilized candidate pathway approach, which has inherited limitations. To overcome the limitations, genome wide approaches, e.g. CGH analysis, have been recently performed. Most common CGH results of SCCHN showed gains at 11q13 which contains PPFIAI, and CCND1, reported (Juhasz et al., 2005; Tan et al., 2008), while frequent losses at 3p, although there are some discrepancies Gollin et al., 2001; Jarvinen et al., 2006; Volker et al., 2010). For the causes of discrepancies in CGH results, ethnical variation, life style, presence of chemotherapy, or regions of SCCHN can be thought. Therefore, we did our best to overcome these limitations with an ethnical population before chemotherapy and a specific region of SCCHN. The present study confirmed losses at 11q13 in 40% of all subjects and identified losses at 3p22~26, which was the most condensed site of CNAs in this study (Fig.1B; Table 2). Interestingly, we observed gains at 8q24, where are located multiple genetic SNP biomarkers for prostate, and breast cancers (Golin et al., 2001). Even though gains on 3q were found less than 40% of all subjects, these alterations were also condensed in the present study. Therefore, we found some common and specific features in CGH results among laryngeal cases.

Focusing on *XPC*, we have studied genetic and phenotypic differences of *XPC* in SCCHN patients from controls: We previously reported not genetic but phenotypic alteration of *XPC* is associated with SCCHN incidence (Yang et al., 2005). A recent cell study, which was obtained from 6 SCC cell lines, also supports our results, i.e. loss of copy number at 3p21-25, which included *XPC*, PPARG, NCKIPSD, MLH1, FANCD2, and CTNNB1 (Tsui et al., 2010).

Concerning genetic alteration and SCCHN progress, a WHO cancer report suggested alteration in the loss of 9p at normal mucosa, 3p and17p at precursor region, 11q, 13q, and14q at dysplasia, 6p, 8q, and 4q at carcinoma for progress of SCCHN (Stewart et al., 2003). In addition, a Japanese group reported SCCHN stage (T1~4) was linked with DNA copy number gain at 3q26~quer (Hashimoto et al., 2001). In the present study, stage 1, 2 or 3 showed somewhat reduced copy number on 3p25.1, compared to stage 4, however, it was not significant. Thus, *XPC* alteration may not affect progress of SCCHN. However, for early detection and therapy as well as prevention of SCCHN, issues of SCCHN progress and genome-wide alteration should be further investigated in enlarged scaled studies.

In addition, environmental and physiological risks for SCCHN, e.g. smoking, alcohol drinking, sex, or age, did not affect amplification on 3p25.1 in this study. Other factors, e.g. HPV infection or promoter methylation at several anticancer genes are recently emphasized as etiology of SCCHN cancer (Bennett et al., 2010). In this study, we did not consider HPV infection due to limitation of HPV diagnosis-information. For the future generation studies, the role of HPV infection on *XPC* or promoter methylation status at *XPC* should be also considered.

In conclusion, we found that the Korean laryngeal SCC had condensed losses on 3p22~26, where contain *XPC* locus. The present study genome- widely confirmed the involvement of *XPC* in occurring of SCCHN, particularly laryngeal SCC. The other genes with loss at 3p22-26 warrant further exploration.

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References

Abbasi R, Ramroth H, Becher H, et al (2009). Laryngeal cancer risk associated with smoking and alcohol consumption is modified by genetic polymorphisms in ERCC5, ERCC6 and RAD23B but not by polymorphisms in five other nucleotide excision repair genes. *Int J Cancer*, **125**,1431-9.

Akervall J (2005). Gene profiling in squamous cell carcinoma of the head and neck. *Cancer Metastasis Rev*, **24**, 87-4.

Bennett KL, Lee W, Lamarre E, et al (2010). HPV status-independent association of alcohol and tobacco exposure or prior radiation therapy with promoter methylation of FUSSEL18, EBF3, IRX1, and SEPT9, but not SLC5A8, in head and neck squamous cell carcinomas. *Genes Chromosomes Cancer*, **49**, 319-26.

Francisco G, Menezes PR, Eluf-Neto J, et al (2008). XPC polymorphisms play a role in tissue-specific carcinogenesis: a meta-analysis. *Eur J Hum Genet*, **16**, 724-34.

Gollin SM (2001). Chromosomal alterations in squamous cell carcinomas of the head and neck: window to the biology of disease. *Head Neck*, **23**, 238-53.

Han W, Han MR, Kang JJ, et al (2006). Genomic alterations identified by array comparative genomic hybridization as prognostic markers in tamoxifen-treated estrogen receptor-positive breast cancer. *BMC Cancer*, **6**, 92.

Hashimoto Y, Oga A, Kawauchi S, et al (2001). Amplification of 3q26 approximately qter correlates with tumor progression in head and neck squamous cell carcinomas. *Cancer Genet Cytogenet*, **129**, 52-6.

Ho T, Li G, Lu J, et al (2007). X-ray repair cross-complementing group 1 (XRCC1) single-nucleotide polymorphisms and the risk of salivary gland carcinomas. *Cancer*, **110**, 318-25.

IARC (2003). Haed and neck cancer. In World Cancer Report, Eds Stewart BW, Kleihues P. IARC Press, Lyon pp232-6.

Jarvinen AK, Autio R, Haapa-Paananen S, et al (2006). Identification of target genes in laryngeal squamous cell carcinoma by high-resolution copy number and gene expression microarray analyses. *Oncogene*, **25**, 6997-7008.

Juhasz A, Balazs M, Sziklay I, et al (2005). Chromosomal imbalances in laryngeal and hypopharyngeal cancers detected by comparative genomic hybridization. *Cytometry A*, **67**,151-60.

Keser I, Toraman AD, Ozbilim G, et al (2008). DNA gains and losses of chromosome in laryngeal squamous cell carcinoma using comparative genomic hybridization. *Yonsei Med J*, **49**, 949-54.

Natasya Naili MN, Hasnita CH, et al (2010). Chromosomal alterations in Malaysian patients with nasopharyngeal carcinoma analyzed by comparative genomic hybridization. *Cancer Genet Cytogenet*, **203**, 309-12.

NTP (2010). Final report on carcinogens background document for formaldehyde. *Rep Carcinog Backgr Doc*, **10**, 5981, i-512.

Patmore HS, Ashman JN, Stafford ND, et al (2007). Genetic analysis of head and neck squamous cell carcinoma using comparative genomic hybridisation identifies specific aberrations associated with laryngeal origin. *Cancer Lett*, **258**, 55-62.

Schlade-Bartusiak K, Stembalska A, Ramsey D (2005). Significant involvement of chromosome 13q deletions in progression of larynx cancer, detected by comparative genomic hybridization. *J Appl Genet*, **46**, 407-13.

Singh B, Wreesmann VB, Pfister D, et al (2002). Chromosomal aberrations in patients with head and neck squamous cell carcinoma do not vary based on severity of tobacco/alcohol exposure. *BMC Genet*, **3**, 22.

Tan KD, Zhu Y, Tan HK, et al (2008). Amplification and overexpression of PPFIA1, a putative 11q13 invasion suppressor gene, in head and neck squamous cell carcinoma. *Genes Chromosomes Cancer* , **47**, 353-62.

Tsui IF, Garnis C (2010). Integrative molecular characterization of head and neck cancer cell model genomes. *Head Neck*, **32**, 1143-60.

Volker HU, Zettl A, Haralambieva E, et al (2010). Leiomyosarcoma of the larynx as a local relapse of squamous cell carcinoma--report of an unusual case. *Head Neck*, **32**, 679-83.

Yang M, Kang MJ, Choi Y, et al (2005). Associations between XPC expression, genotype, and the risk of head and neck cancer. *Environ Mol Mutagen*, **45**, 374-9.