

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 polymorphisms (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., Minneapolis, 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.ucsc.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 metalloproteinase 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 × 100 Loss, Normalized log₂-Ratio <-0.25; loss %, No. of lost subjects/No. of all subjects ×100; 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 PPFIA1, and CCND1, reported (Juhász 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 *CTNBN1* (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 and 17p at precursor region, 11q, 13q, and 14q 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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