FREQUENT GAIN OF THE p40/p51/p63 GENE LOCUS IN PRIMARY HEAD AND NECK SQUAMOUS CELL CARCINOMA

Kengo Yamaguchi1,2, Li Wu1, Otavia L. Caballero1, Kenji Hibi1, Barry Trink1, Vicente Resto1, Paul Cairns1, Kenji Okami1, Wayne M. Koch1, David Sidransky1 and Jin Jen1*

1Division of Head and Neck Cancer Research, Department of Otolaryngology, Head and Neck Surgery, The Johns Hopkins University, Baltimore, Maryland, USA
2Department of Otolaryngology, National Defense Medical College, Tokorozawa, Japan

We have identified a new human p53 homologue, p40 (p51/p63). This gene was mapped to the distal arm of 3q and was found to be essential for normal epithelial development. We used microsatellite and FISH analyses to search for genetic alterations of p40 in primary HNSCC. A more precise localization of p40 was completed using 6 known markers on 3q and a newly isolated microsatellite marker within the p40 gene. We also determined the genomic organization of the p40 gene using human YAC and BAC clones. We then localized a new microsatellite marker within the chromosome 3 centromere. We found frequent copy number increase at the p40 locus in HNSCC, indicating an important role for p40/p51/p63 amplification in the development of this cancer type. Our results further support the notion that this gene plays an oncogenic role in tumor development.

MATERIAL AND METHODS

Patients. DNA extraction for microsatellite analysis and sample preparation for FISH

Twenty-six HNSCC were collected and stored at −80°C following surgical resection with prior consent from Johns Hopkins Hospital patients. For DNA extraction, the tumor specimens were microdissected on a cryostat to obtain greater than 70% neoplastic cells. The sections were digested with SDS/proteinase K, and DNA was extracted by phenol-chloroform followed by ethanol precipitation (Maniatis et al., 1989). Paired normal DNA was obtained in the same manner as the tumor sample from peripheral lymphocytes or normal tissues. Fifty-micrometer-thick frozen sections were cut out for FISH analysis followed by interphase nucleus preparation (see below). Normal lung tissue was prepared in the same manner as a negative control, and head and neck cancer cell lines known to have p40 amplification were used as positive controls (Hibi et al., 2000).

Identification of BAC clones containing the p40 gene

Two overlapping BAC (bacterial artificial chromosome) clones were isolated from a human genomic BAC library (Genome Systems, St. Louis, MO). PCR primers were designed from the p40 cDNA sequences (GenBank accession AF061512) and were used to isolate BAC#10102 and BAC080e1. These 2 BACs were amplified from the host bacteria, analyzed by direct sequencing to establish the genomic organization of p40, and further mapped by microsatellite markers for detailed chromosomal mapping of p40.

Isolation of a novel microsatellite marker

The method for isolation of microsatellite markers from BAC has been previously described in detail (Cairns et al., 1997). Briefly, the two BACs were subcloned into Bluescript after diges-
tion with Sau3A1 and plated. Ampicillin-resistant colonies were lifted onto nylon membranes and then screened by hybridization with a radiolabeled (GT)_{10} oligomer probe. The new microsatellite locus isolated from BAC80eo1 was designated D3S3540, and the primer sequences used for PCR amplification were:

\[
F, 5'-GACTCGTGGCCAATTAGCC-3' \\
R, 5'-CAAGGGGACGGAATCTGACG-3'
\]

These primers produce a product of 152 bp in size, and the marker was found to be informative in approximately 72% of cases after examining 55 non-related individuals.

Mapping of p40/p51/p63 using microsatellite markers and YAC contigs

To determine the precise genomic location of p40/p51/p63, 4 yeast artificial chromosome (YAC) clones and the 2 p40 BAC clones were tested by PCR amplification using microsatellite and STS (sequence-tagged site) markers. Four YACs [955B2, 913D2, 889A7 and 749E5] were amplified YACs 955B2 and 913D2 but not 889A7 and 749E5. p40/p51/p63 lies between the markers WI-1189 and D3S3422.

| TABLE 1 – INTRON/EXON BOUNDARIES OF P40 |
|-----------------------------------------|
| Exon 5' positions | Intron5' | exon . . 3' exon/intron |
| 1 334 | aagagagagatttctggttcttgcttatc/TTCTTTAA | . . TAGTGAAG/taaaggtttagttttagcactccattt |
| 2 478 | taattttggttttcggtctcttcgcag/CAGAAGT | . . GTGACTG/taaagaggacgggccgactcactgac |
| 3 733 | tcttttcacggtgaaactcttaagag/TATTCCCA | . . AAGGAGG/gtaaagaggacgggccgactcactgac |
| 4 920 | tattaatttttctgtctcgcag/AGACAT | . . ACCCAGAG/gtaaagggacgggccgactcactgac |
| 5 1,036 | taaccaagagatttctgtctcgcag/GTTGCGA | . . CCAGAGAG/taaagagcagcgggcacgacatactgac |
| 6 1,146 | ctgttttttttattactcctcag/TGGGGCA | . . AAAGGCG/gtaaagagcagcgggccgactcactgac |
| 7 1,283 | sggctgagcaggttcttcacccacacag/CATTTCG | . . TATTCCAG/gtaaagagcagcgggccgactcactgac |
| 8 1,367 | cttttcttcctctctctgtctctcatorag/TGGGGCA | . . AAAGGCG/taaagagcagcgggccgactcactgac |

1Positions of nucleotides are according to the cDNA sequence deposited in GenBank (accession AF061512).

FIGURE 1 – Detailed mapping of p40/p51/p63 on an integrated map. D3S3540 was isolated from BAC80eo1 by screening a subcloned fragment with a radiolabeled (GT)_{10} oligomer. Two BACs overlap at exon 2 of p40. Three STSs of p40/p51/p63 and D3S3540 amplified YACs 955B2 and 913D2 but not 889A7 and 749E5. p40/p51/p63 lies between the markers WI-1189 and D3S3422.

FIGURE 2 – Genomic organization of p40 and other transcripts. p40 is the shortest transcript and has the same N-terminus as ΔNp63αβγ. Exons 2 through 8 of p40 are equivalent to exons 4 through 10 of p51 and p63. The 3' UTR for p40 is the same as intronic sequence between exons 10 and 11 of p51 and p63.
dehydrated. The chromosomes were denatured for 5 min in 70% PBS-buffered 1% formaldehyde for 5 min and again ethanol dehydration, nuclei attached to a silane-coated slide were fixed in 

FISH analysis

when compared with the same alleles in the control. 

were scored if the allelic ratio differed more than 30% in tumor cases, the ratio of the upper and the lower alleles in tumor DNA 

markers used in this study were D3S3596, D3S3628, D3S3649, 

were determined by direct 

Microsatellite analysis for allelic imbalances

and normal was calculated using NIH image 1.58f (Scientific 

CCCAG-3'. The 4 YAC clones were verified to contain the locus-specific markers D3S3549 and/or D3S3530. 

Genomic organization of p40

The intron/exon boundaries of p40 were determined by direct sequencing of the BAC genomic DNA and by the divergence between the BAC genomic and the cDNA sequences. 

Microsatellite analysis for allelic imbalances

Matched DNA from tumor and normal control was examined for allelic imbalance by PCR-based microsatellite analysis as described previously (Califano et al., 1996). The microsatellite markers used in this study were D3S3596, D3S3628, D3S3649, D3S3530, D3S3540, D3S1305 and D3S1311. For informative cases, the ratio of the upper and the lower alleles in tumor DNA and normal was calculated using NIH image 1.58f (Scientific Computing Resources Center, Bethesda, MD), and imbalances were scored if the allelic ratio differed more than 30% in tumor when compared with the same alleles in the control. 

FISH analysis

From each specimen, a single-cell nuclei suspension was prepared by proteolytic digestion with pepsin at a concentration of 0.25 mg/ml in 0.01N HCl for 15 min at 37°C. After ethanol dehydration, nuclei attached to a silane-coated slide were fixed in PBS-buffered 1% formaldehyde for 5 min and again ethanol dehydrated. The chromosomes were denatured for 5 min in 70% formamide and 2× SSC (pH 7.0) followed by dehydration in cold ethanol. The BAC80eo1 clone was used as the p40/p51/p63 probe and was labeled by nick translation with digoxigenin-11-dUTP. The biotin-labeled centromeric probe (locus D3Z1) was purchased (Oncor, Gaithersburg, MD). The p40/p51/p63 probe (10 ng/µl) was denatured at 75°C for 5 min and allowed to pre-anneal at 37°C for 15 min with 1 µg/µl of both Cot-1 DNA and salmon sperm DNA in 65% formamide, 10% dextran sulfate and 2× SSC. After denaturation, the centromeric probe was added and hybridized overnight to the nuclei at 37°C. Slides were washed twice in 1× SSC at 72°C for 5 min followed by a blocking step with phosphate Nonidet buffer 5% non-fat dry milk for 10 min. To detect the p40/p51/p63 probe, slides were incubated with series of mouse anti-DIG, rabbit anti-mouse-TRITC-conjugate, and goat anti-rabbit-TRITC conjugate for 30 min each at 37°C. Slides were also incubated with FITC-conjugated avidin, biotin-conjugated goat anti-avidin and again FITC-conjugated avidin for 40 min, 10 min and 10 min at 37°C to detect the centromeric probe. Nuclei were counterstained with DAPI and examined under a Zeiss Axiphot epifluorescence microscope. Signals close to one another were counted as one signal because they probably represented sister chromatids in S or G2 phase. The number of p40 and centromeric signals were counted in 200 non-overlapping nuclei per slide. 

RESULTS

Mapping of p40

Fine physical mapping of p40/p51/p63 was performed on a human genomic YAC and BAC contig. Three STSs and D3S3540 (isolated from BAC80eo1) were successfully amplified on YACs 955B2 and 913D2 but not on 889A7 or 749E5 (Fig. 1). These findings confirmed an earlier report that localized p51 centromeric to marker WI-1189 (Osada et al., 1998) and now place p40/p51/p63 between WI-1189 and D3S3422. The STS from exon 2 of p40 was amplified from both BACs, indicating a region of overlap between the BACs. An STS from exon 8 of p40 amplified on BAC80eo1 but not on BAC10102, and an STS from exon 2 of p51ab amplified BAC10102, not BAC80eo1. Figure 1 summarizes our results and integrates our data with currently available data from several sources (Genome Database; Genethon, Whitehead Institute for Biomedical Research/MIT Center for Genome Research, Cambridge, MA).

Genomic organization

The intron/exon boundaries of p40 were determined by direct sequencing of the BAC insert and subsequent comparison to the
previously published p40 cDNA sequence (Trink et al., 1998). Eight coding exons were identified. The intron/exon boundaries are described in Table I. We previously identified p40 as a smaller transcript of DNp63abg. Both of these transcripts lack the putative transactivation domain described for p51ab and TAp63abg. This domain is created by a splice variant that replaces exon 1 of p40 with an alternative 5' end. The cDNA region corresponding to the DNA binding domain of p53 is encompassed from exon 2 to exon 6. Exon 7 contains a putative oligomerization domain similar to the p53 oligomerization domain. As shown in Figure 2, p40 has the same N-terminus as ΔNp63αβγ. The intron/exon boundaries for exons 1 through 8 of p40 were essentially identical to p63 and p51 (Tani et al., 1999). All other transcripts have additional exons at the 3' end.

Allelic imbalances of the p40/p51/p63 locus

We used 7 microsatellite markers (6 known and the new marker D3S3540) to assess allelic balance in primary tumors. Fourteen of 26 HNSCC (54%) showed allelic imbalance in at least one microsatellite marker (Fig. 3). Three tumors—2, 6 and 9—demonstrated severe imbalances with an allelic ratio difference of 70% to 100%. Tumor 1 (Fig. 4) demonstrates a typical imbalance (allelic

![Figure 4](image1.png)

**Figure 4**—Microsatellite analysis in primary HNSCC. (a) Tumor 1 shows a new allele at D3S3628 (microsatellite instability) and mild (allelic ratio difference 30%–70%) allelic imbalance at D3S3530, D3S3540 and D3S1305. (b) Severe allelic imbalance in tumor 2 (allelic ratio difference 85%–100%). (c,d) Lack of imbalance at all informative microsatellite loci in tumor 15 and 16, respectively. Allelic imbalances were scored using densitometric calculation if the allelic ratio was decreased more than 30% in the tumor when compared with the same allele in the control. N, normal DNA; T, tumor DNA.

![Figure 5](image2.png)

**Figure 5**—Analysis of HNSCC by fluorescence in situ hybridization (FISH). Dual-color FISH with probes for chromosome 3 and p40. Interphase nuclei were prepared from 50-μm-thick sections. Signals close to one another were counted as a single signal because they probably represented sister chromatids in S or G2 phase. (a) Tumor 1 with allelic imbalance demonstrating 2 centromeric signals (in green) and 4 p40 signals (in red) in the right nucleus. (b) Tumor 15 with balanced allele showing multiple signals of p40 and 2 centromeric signals in the left nucleus. Scale bar = 20 μm.
ratio difference of 30% to 70%), while tumor 2 shows the most severe imbalances in this set of tumors (allelic ratio difference 85% to 100%). The remaining 12 of 26 tumors had retention of both alleles without evidence of imbalance (see Fig. 4, tumors 15 and 16).

Detection of increased p40/p51/p63 signal by FISH

Out of 26 HNSCC evaluated by microsatellite analysis, fresh frozen tissue was available in 13 tumors for FISH analysis. The FISH results and allelic status determined by microsatellite analysis are shown in Table II. All neoplasms with allelic imbalance, except tumor 3, demonstrated an apparent increase in p40/p51/p63 signals. A majority of the tumors without allelic imbalance at p40 (D3S3540) and the surrounding markers exhibited polysomy of chromosome 3q. Interestingly, tumors 15 and 16 demonstrated amplification of the p40 locus. There was no correlation between allelic status and copy number. For tumor 15, the p40 marker was not informative, and high gains in copy number could not be scored by conventional microsatellite analysis at saturation (35 cycles). However, PCR with this marker below saturation (18–25 cycles) consistently yielded higher signal in tumor DNA than control DNA, suggesting an increase in copy number at p40 identified by FISH (data not shown). Typical nuclei from tumors 1 and 15 are shown in Figure 5.

DISCUSSION

Current studies suggest that p40/p51/p63 may encode transcripts with distinct functions, from suppression of growth to enhancement of growth. We sought to precisely localize p40 and integrate its location on known physical maps by isolating a novel microsatellite marker from a BAC clone containing p40 and testing the locus compared with 6 other markers within a 19-cM genetic interval surrounding the gene. Fifty-four percent of 26 primary HNSCC showed allelic imbalance in at least one of these microsatellite loci, and a few cases had localized allelic imbalance within this region.

The frequency of allelic imbalance of the p40/p51/p63 genomic locus in our study is markedly lower than previously reported values of 3q26-qter gain, which have ranged from 77% to 83% (Speicher et al., 1995; Bockmühl et al., 1996). This discrepancy...
prompted us to carry out FISH analysis of primary tumor samples in order to better assess the true genomic dosage of 3q26-qter. FISH has been shown to be a more quantitative technique than microsatellite analysis and a more sensitive technique than CGH. FISH analysis revealed that all 13 tumors tested had an increased p40 signal regardless of allelic status. We also observed that an increased chromosome 3 centromeric signal was often accompanied by p40 gain, suggesting whole 3q duplication. However, 5 tumors had higher p40 copy number than the centromere, indicating the presence of more specific amplification of distal 3q including p40. These data are consistent with an increased ratio of 3q26 to 3q27 by CGH analysis (Speicher et al., 1995; Bockmühl et al., 1996) and the finding that a region at 3q25-qter of intact human chromosome 3 is regularly retained after several passages in microcell hybrid lines (Imreh et al., 1997). Several genes associated with oncogenic function have been mapped to distal 3q, including the telomerase RNA gene (hTR) at 3q26.3, BCHE and SLCA2A2 at 3q26 (Brass et al., 1997b), elf-4 gamma gene at 3q26.1-3 (Brass et al., 1997a) and PIK3CA at 3q26 (Shayesteh et al., 1999). A single-copy number of elf-4TR copy number by FISH was detected in 97% of primary HNSCC and carcinomas of the uterine cervix (Soder et al., 1997).

Our results show that increased copy number by FISH in a region of interest does not always exhibit allelic imbalance by microsatellite or DNA RFLP analysis in cancer cells, whereas good correlation has been shown at regions with LOH (Okami et al., 1997). In previous studies, 2 of 5 ductal carcinomas in situ of breast with increased centromeric signal of chromosome 17 (Murphy et al., 1995) and 1 of 8 renal cell carcinomas with an increase of chromosome 8 (Hughson et al., 1998) retained balanced alleles, suggesting that the discrepancy was caused by tetrasomy equally derived from each original homologous pair of chromosomes. In a separate study, 18 of 50 overrepresented genomic regions in 3 human testicular germ-cell tumor cell lines also retained heterozygosity (Al-Jehani et al., 1995). A possible explanation for our observation that the p40 signal did not correlate at all with allelic status is suggested by the model shown in Figure 6. This occurs when duplication of whole 3q derives equally from each homologous chromosome, thus retaining allelic balance. Allelic imbalance by specific amplification can be masked by the duplication.

In our study, tumor 2 showed complete allelic loss, whereas FISH analysis revealed a single copy increase at the p40 locus, suggesting that this allelic loss resulted from recombination or chromosome loss and duplication. However, our data generally indicate that, in HNSCC, the p40 locus is not a target of deletion. The latter result is further substantiated by several reports that now show that p40 is rarely mutated in cancer cells (Osaada et al., 1998; Tani et al., 1999; Hagiwara et al., 1999). Rather, gain of the 3q locus is very common in HNSCC and correlates with p40 overexpression at the RNA and protein level in primary squamous cell carcinoma of the lung and HNSCC cell lines (Hibi, et al., 2000). Our data support the notion that this gene plays an oncogenic role in tumor development. Precise characterization of this genetic change will provide a better understanding of how these cancers arise and the biochemical pathways that lead to their progression.

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