Interrogation of Chromosome 13q12-14 in Esophageal Squamous Cell Carcinoma

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Abstract: Previous studies of esophageal squamous cell carcinoma (ESCC) suggested chromosome region 13q12-14 harbors a familial ESCC gene. DNA sequencing of the BRCA2 gene, located on 13q12, showed evidence of both germline and tumor specific alterations but the frequency of changes was low and did not fit the classic Knudsen two-hit gene inactivation model. To further investigate chromosome 13q12-14 in ESCC, quantitative expression measurements were performed on BRCA2 and 11 neighboring genes in matched normal epithelium and tumor from 17 cases. Transcript analysis showed normal levels of five genes, tumor down-regulation of two genes (TNFRS19 and TPT1), and tumor up-regulation of five genes, including BRCA2. No evidence of BRCA2 loss-of-function was detected based on reduced mRNA in tumor cells. Between 13q12.3 (KATNAL1) and 13q12.3-q13 (CCNA1) five adjacent genes showed increased mRNA expression raising the possibility of a DNA amplicon; however, qPCR analysis showed normal DNA amounts in this region. CCNA1 transcript was significantly up-regulated in tumors and was thus further interrogated at the protein level by immunohistochemistry. CCNA1 staining was restricted to normal basal epithelium and was not expressed in more superficial, differentiated regions. In contrast, the CCNA1 protein was ubiquitously and highly expressed throughout tumor foci. Overall, these data from a relatively small number of cases (17) suggest that TNFRS19 and TPT1 deserve further investigation as candidate tumor suppressor genes in esophageal cancer in a larger patient series; BRCA2 mRNA is increased in the tumors, likely as a compensatory response to the marked DNA damage that is present in these lesions; and, CCNA1 was identified as a novel up-regulated gene in ESCC.

Keywords: Esophagus, carcinoma, squamous cell, chromosome 13q, BRCA2, genomic region, gene expression, upregulated region, microdissected tissue.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the fifth leading cause of cancer death in the world, with a significantly higher incidence in certain geographic areas such as Shanxi Province in China. Epidemiological studies suggest that both environment and genetics influence ESCC development and there is evidence for Mendelian inheritance of a tumor suppressor gene (TSG) [1, 2]. Genomic studies of ESCC have identified several chromosomal regions with high levels of loss of heterozygosity (LOH), including chromosome 13 [1-3]. Additionally, a statistically significant difference in allelic loss on chromosome 13 was observed between sporadic versus familial cases suggesting this chromosome may harbor an inherited TSG [4, 5].

ESCC shows extensive chromosomal instability thus it has not been possible to precisely map a minimal deletion interval; however, both the tumor LOH data and the results comparing allelic loss in sporadic versus familial cases point to the genomic region spanning chromosome 13q12-14 as the most likely location of a putative esophageal cancer gene [6-11]. Direct DNA sequencing of selected candidates to date has not identified a classically inactivated TSG on chromosome 13, although the data on BRCA2 are difficult to interpret as missense mutations have been identified in over 10% of tumors, and in the germline of 5% of patients with a family history of ESCC [12-14]. Moreover, similar results of
BRCA2 germline mutations have been observed in high-risk ESCC populations in both Iran and India [15, 16]. One strategy to assess the status of candidate TSGs in a genomic interval of interest is to evaluate transcript levels. Alterations in mRNA in tumors can occur due to several different mechanisms, including homozygous DNA deletion, aberrant epigenetic regulation, decreased dosage due to haplinsufficiency, or decreased stability of mutated mRNAs [17]. In the present study we performed quantitative RT-PCR (qRT-PCR) analysis on 12 genes on 13q12-14, including BRCA2, to assess if any of the candidates exhibited loss-of-function based on reduced expression of mRNA. The gene set was selected to include genomic locations across the region of interest on chromosome 13, and genes with potential growth related functions.

MATERIALS AND METHODS

Clinical Tissue Specimens

Twenty-four cases comprising 52 frozen tissue blocks of newly diagnosed and untreated human esophageal squamous cell carcinoma (ESCC) were obtained from patients who underwent esophageal resection at Shanxi Cancer Hospital and Institute, Taiyuan in Shanxi Province of China. The specimens were collected under an IRB-approved protocol and transferred to the National Cancer Institute (NCI). The tissues were evaluated by a pathologist (JRC) and blocks from seventeen cases were selected for study based upon both histologic criteria (presence of ESCC epithelium and matched normal squamous epithelium) and microdissection criteria (>10,000 normal epithelial or tumor cells).

Tissue Microdissection

Matched normal epithelium and tumor were dissected from histological sections using laser capture microdissection (LCM) as described previously [18]. Frozen tissue samples were hematoxylin and eosin (H&E) stained prior to LCM and approximately 10,000 cells were procured from each block (equivalent to ~3,000 LCM shots per tissue type), immediately placed into lysis buffer, and stored at -80°C until RNA extraction. A replicate microdissection of all tissues was conducted using a pathologist (JRC) and blocks from seventeen cases were selected for study based upon both histologic criteria (presence of ESCC epithelium and matched normal squamous epithelium) and microdissection criteria (>10,000 normal epithelial or tumor cells).

RNA Isolation, Quantitation, and Qualitation

RNA extraction and isolation from samples were conducted as previously described [19, 20]. Following RNA isolation, 3 μl aliquots per sample were taken for RNA quantity and quality measurement and used immediately. All remaining RNA samples were stored at -80°C. Quantitation and qualification of individual sample total RNA was conducted using NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) equipment, respectively. Sample quality was assessed by RNA integrity number (RIN) using 2100 Expert software (Agilent Technologies, Inc.).

Chromosome 13q Genes

Twelve genes spanning 27.5 million bp between 13q11-13q14 were selected for analysis (Fig. 1, Table 1). The genes were chosen such that they spanned the genomic region of interest on chromosome 13 and because reports in the literature suggested a potential role in cell growth [3, 8, 21, 22].

![Fig. (1). Schematic of chromosome 13 showing the location of the twelve genes that were analyzed by qRT-PCR in patient-matched normal and tumor samples.](image-url)
Quantitative PCR (qPCR) DNA Measurement

qPCR analysis was performed in triplicate singleplex reactions as previously described [19] using the extracted tumor and matched normal DNA samples. TaqMan primer/probe sets for genes of interest were negative in all runs. Relative quantitation analysis of gene expression data was performed according to the 2^-ΔΔCT method, with mean biologic fold change calculated directly from mean ΔΔCT value [24].

DNA Isolation, Quantitation, and Qualitation

DNA extraction and isolation was conducted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Cat # 69504) according to manufacturer’s protocol. DNA quantity and quality assessment was conducted using NanoDrop (NanoDrop Technologies) and Bioanalyzer (Agilent Technologies, Inc.) equipment, respectively, according to the manufacturer’s protocol.

Table 1. 13q Genes Evaluated by qRT-PCR Analysis of Microdissected ESCC Matched N and T Tissues

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene Name</th>
<th>Map</th>
<th>Category</th>
<th>Gene Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>LATS2</td>
<td>Large tumor suppressor, homolog 2</td>
<td>13q11-1q12</td>
<td>Kinase</td>
<td>Protein kinase</td>
</tr>
<tr>
<td>EFHA1</td>
<td>EF-hand domain family, member A1</td>
<td>13q12.11</td>
<td>Select calcium binding protein</td>
<td>Calmodulin related protein</td>
</tr>
<tr>
<td>TNFRSF19*</td>
<td>tumor necrosis factor receptor superfamily, member 19</td>
<td>13q12.11-12q3</td>
<td>Receptor</td>
<td>Cytokine receptor</td>
</tr>
<tr>
<td>RNF6</td>
<td>ring finger protein (CHC3 type)</td>
<td>6q22.11-12.3</td>
<td>Transcription factor</td>
<td>Transcription cofactor</td>
</tr>
<tr>
<td>KATNAL1</td>
<td>katanin p60 subunit A-like 1</td>
<td>13q12.3</td>
<td>Cytoskeletal Protein</td>
<td>Microtubule family cytoskeletal protein</td>
</tr>
<tr>
<td>HSP1</td>
<td>heat shock protein</td>
<td>13q12.3</td>
<td>Chaperone</td>
<td>Hsp 70 family chaperone</td>
</tr>
<tr>
<td>BRCA2*</td>
<td>breast cancer 2, early onset</td>
<td>13q12.3</td>
<td>Nucleic acid binding</td>
<td>Damaged DNA-binding protein</td>
</tr>
<tr>
<td>RFC3</td>
<td>replication factor C (activator 1)</td>
<td>13q12.3-12q13</td>
<td>Nucleic acid binding</td>
<td>DNA-directed DNA polymerase</td>
</tr>
<tr>
<td>CCNA1**</td>
<td>cyclin A1</td>
<td>13q12.3-12q13</td>
<td>Select regulatory molecule</td>
<td>Kinase modulator</td>
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<td>ELF1</td>
<td>E74-like factor 1 (ets domain transcription factor)</td>
<td>13q14.11</td>
<td>Transcription factor</td>
<td>Other transcription factor</td>
</tr>
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<td>TPT1</td>
<td>tumor protein, translationally-controlled 1</td>
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<td>Cytoskeletal protein</td>
<td>Microtubule family cytoskeletal protein</td>
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<tr>
<td>RB1</td>
<td>retinoblastoma 1</td>
<td>13q14</td>
<td>Transcription factor</td>
<td>Other transcription factor</td>
</tr>
</tbody>
</table>

*Evaluated by qRT-PCR (RNA) and qPCR (DNA).
**Evaluated by qRT-PCR (RNA), qPCR (DNA), and IHC (protein).

was conducted using cDNA from a replicate LCM sample. Negligible contamination (0.1%) of genomic DNA was confirmed by the observation of a cycle threshold (CT) of 10 comparing RT-negative with RT-positive samples. Triplicate qPCR assays were performed following RT. Controls consisting of total human esophagus RNA (~12ng/ul; Ambion, Austin, TX, USA) were positive in all runs, and no-template controls consisting of sterile molecular grade water were negative in all runs. Relative quantitation analysis of gene expression data was performed according to the 2^-ΔΔCT method, with mean biologic fold change calculated directly from mean ΔΔCT value [24].

Statistical Analysis of Gene Expression

We tested for BRCA2 and CCNA1 up-regulation and TNFRSF19 and TPT1 down-regulation using a two-sided paired t-test and Bonferroni multiple correction. The mean difference in CT values by spatial location of the genes were also analyzed and plotted for LATS2, EFHA1, TNFRSF19, RNF6, KATNAL1, HSP1, average of 3 BRCA2 markers, RFC3, CCNA1, ELF1, TPT1, RB1. Log-base 10 P-values for two-sided t-tests comparing differences in the CT values across successive genes (located next to each other) were analyzed with plots demonstrating the log p-value for a test of whether there is a shift between the ith and (i-1)th gene. A small p-value reflects evidence of a shift in mean values between the two markers. Bonferroni multiple comparisons correction was applied and statistical significance was considered a p-value less than 0.05/11=0.0045 (accounting for 11 comparisons with a Bonferroni multiple comparisons correction).

Immunohistochemistry (IHC)

One case of formalin-fixed, paraffin-embedded tissue (FFPE) was immunohistochemically stained using biotinylated link streptavidin HRP (LSAB2, DAKO, K0675). A deparaffinized slide containing both normal epithelium and ESCC was heat-treated using a steamer (Black and Decker, HS2000) in Tris/EDTA buffer, Ph9 (DAKO, S2367) for 20 minutes unmasking cyclin A epitopes. The slides were left in solution for addition 20 minutes to cool to room temperature and treated with peroxidase-blocking reagent (DAKO, S2001) to inhibit endogenous peroxidase activity. The sections were then incubated with a monoclonal anti-CCNA1 antibody (NCL-cyclin A, clone 6E6, 1:50 at room temperature). Afterwards,
they were incubated with the biotinylated link antibody and streptavidin–peroxidase for 15 minutes each (DAKO, K0675). Subsequently, liquid DAB (diaminobenzidine) (DAKO, K3466) was applied for 5 minutes and the sections counterstained in hematoxylin (Lerner-2 Laboratories, Cat# 1931413). Slides were then dehydrated and mounted with Cytoseal XYL (Richard-Allan, Cat# 8312-4) mounting medium. Biotinylated link strepavidin HRP were prediluted visualization kits from DAKO, and anti-CCNA1 primary antibody was diluted with ready-to-use diluent (DAKO, S0809). CCNA1 expression in tissues was evaluated by IHC analysis using the DAKO Autostainer Universal Staining System (DAKO, S3800).

RESULTS

Quantitative RT-PCR and qPCR Interrogation of Chromosome 13q12-14

Transcript expression of 12 genes within the 13q LOH region was analyzed by qRT-PCR using matched normal epithelium and squamous cell carcinoma from 17 cases (Fig. 1, Table 1). Full-thickness microdissection of the epithelium was performed for normal cell populations, and islands of invasive tumor were procured for the cancer samples. The RNA quantity was measured by NanoDrop (average tumor = 20.42 ng/µl; average normal = 16.59 ng/µl) and the RNA quality was determined by BioAnalyzer (average tumor = 4.5 RIN; average normal = 5.1 RIN). For a normalization strategy, three steps were utilized: cell count during microdissection; total RNA measurement; and the use of a tissue specific endogenous housekeeping gene [19, 20].

Overall, five of the 12 transcripts were up-regulated in the majority of tumors (KATNAL1, HSP1, BRCA2, RFC3, CCNA1), two were down-regulated (TNFRSF19, TPT1), and five showed no consistent change (Table 2). The BRCA2 mRNA was increased an average of 5.02-fold in the ESCC samples and thus did not show evidence of loss-of function based on reduced transcript expression. CCNA1, a cell cycle regulatory gene, showed an increase of 129.36-fold, the most of any of the genes analyzed. The increases in BRCA2 and CCNA1 were both statistically significant (P<0.001).

The two down-regulated genes, TNFRSF19 and TPT1, showed a greater than 1.5 fold decrease in mRNA levels in 10 of 17 and 11 of 17 tumors, respectively (Table 2). Overall, TNFRSF19 showed an average decrease in the tumors of 2.31 fold, and TPT1 had a mean decrease of 1.66. Although a trend toward decreased TNFRSF19 and TPT1 transcript levels was observed in ESCC, these results were not statistically significant due in part to the relatively small number of cases analyzed.

Table 2. Individual Case ΔΔCT Values of 13q Region Gene Expression

<table>
<thead>
<tr>
<th>Case</th>
<th>LATS2</th>
<th>EFHA1</th>
<th>TNFRSF19</th>
<th>RNF6</th>
<th>KATNAL1</th>
<th>HSP1</th>
<th>BRCA2*</th>
<th>RFC3</th>
<th>CCNA1</th>
<th>ELF1</th>
<th>TPT1</th>
<th>RB1</th>
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<td>1</td>
<td>2.88</td>
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<td>0.63</td>
<td>-0.12</td>
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<td>1.60</td>
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<td>-0.04</td>
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<td>-0.66</td>
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<td>-2.72</td>
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<td>2.11</td>
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<tr>
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<td>0.94</td>
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<td>-0.15</td>
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<tr>
<td>Mean ΔΔCT</td>
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<td>129.39</td>
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</table>

*BRCA2* is the average of three values: two replicate tissue dissections and one additional exon location.
Light blue = >1.5 fold change down-regulation of gene expression in tumor.
Light yellow = 1.5 < x < 20 fold change up-regulation of gene expression in tumor.
Bright yellow = > 20 fold change up-regulation of gene expression in tumor.
Mean fold change calculated from average ΔΔCT value of all cases per gene.
Table 3, Summary of 13q Gene Expression of Seventeen Cases of Microdissected Frozen ESCC Tissues and Matched Normal Tissues

<table>
<thead>
<tr>
<th>Gene</th>
<th>Map Value</th>
<th>Start (bp)</th>
<th>Stop (bp)</th>
<th>Size (bp)</th>
<th>Mean ΔACT</th>
<th>S.D.</th>
<th>Mean Fold Change*</th>
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<td>BRCA2</td>
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<td>RFC3</td>
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<td>35915008</td>
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<td>ELF1</td>
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<tr>
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<td>2.05</td>
<td>-1.08</td>
</tr>
</tbody>
</table>

Genes without arrow designation had no change in expression of tumor compared to normal (<1.5 fold change).

1Down-regulation of gene expression in tumor tissue (T<N).
2Up-regulation of gene expression in tumor tissue (T>N).
*4+ (>20 fold) Up-regulation of gene expression in tumor tissue (T>N).

The region spanning KATNAL1 to CCNA1 on chromosome 13 demonstrated up-regulation of all five genes in the interval (Table 2) raising the possibility of a DNA amplicon that could involve BRCA2 or known growth related genes such as CCNA1. Therefore, a statistical analysis was conducted using the qRT-PCR transcript expression data of the 12 genes in the region (Fig. 2). Significant mean shifts were observed between RNF6 and KATNAL1 (down, P-value=4.19E-03) and between CCNA1 and ELF1 (up, P-value=1.19E-06), demarcating the region of consecutive gene up-regulation. Since the analysis of increased gene expression in this interval showed statistical significance, we measured DNA content at the TNFRSF19, BRCA2, and CCNA1 genes using qPCR. No evidence of DNA amplification across the region of the five up-regulated genes was observed (data not shown).

Immunohistochemical Analysis of CCNA1

CCNA1 transcript was markedly elevated in ESCC and a commercial antibody for IHC was available, thus we qualitatively analyzed CCNA1 protein expression and distribution in normal and tumor cells. IHC analysis was conducted using formalin-fixed, paraffin-embedded tissue from one case in the study. The staining showed CCNA1 nuclear positivity in the basal region of normal epithelium where cell division occurs, but little to no expression in the more differentiated and superficial areas (Fig. 3). In contrast, both dysplastic epithelium and tumor showed CCNA1 staining throughout the lesions, consistent with cell division occurring widely in the transformed cell population. These results suggest that CCNA1 protein expression is associated with cell division in both normal and transformed cells.

DISCUSSION

The incidence of ESCC is notably high in several geographic regions of the world including Shanxi Province in China. At present, the etiology of this phenomenon is not known although both genetic and environment influences are suspected to play a role [1, 2]. The BRCA2 gene on chromosome 13q12 may be involved in ESCC based on four lines of evidence: chromosome 13 is frequently deleted in this cancer type; there is a statistically significant difference in LOH on chromosome 13q12 between family history positive and family history negative cases; mutations in BRCA2 are observed in a subset of tumors; and, germline mutations are present in affected kindreds [3-5, 13, 21, 22]. However, to date the exact role of the BRCA2 gene in ESCC is unclear as the frequency of tumor-specific and germline mutations is low, and the pattern does not fit the classic Knudsen two-hit model [5, 12-14].

To further investigate BRCA2 and other genes on 13q12-14, we examined expression levels in matched normal esophageal squamous epithelium and ESCC from seventeen patients using qRT-PCR. Overall, there were three major findings in the study. No evidence of loss of function of the BRCA2 gene was observed based on reduced mRNA levels; CCNA1 was identified as a novel up-regulated gene in ESCC; and, the TNFRSF19 and TPT1 transcripts were reduced in the majority of tumors studied.

The increased levels of BRCA2 mRNA in ESCC likely represent a compensatory change in response to the extensive genomic instability that is observed in this tumor type. BRCA2 functions as a DNA repair enzyme via interaction with the RAD51 protein that is known to correct double-stranded DNA breaks and regulate homologous recombination during meiosis [25-29]. Loss-of-function of BRCA2 is associated with several different tumor types with pronounced genomic instability. Further study of the role of BRCA2 is warranted in order to both understand the role of the gene in ESCC and to evaluate its potential implications for treatment as inhibition of the compensatory cellular DNA repair response may be an effective therapeutic strategy by
making tumor cells susceptible to DNA damaging drugs [30-34].

The CCNA1 gene is located on chromosome 13q12.3-13 and encodes a protein expressed in testis, brain and several leukemic cell lines that is thought to primarily function in the control of the meiotic cell cycle [35, 36]. CCNA1 protein binds both CDK2 and CDC2 kinases, giving it two distinct kinase-related activities, one appearing in S phase, the other in G2, and thus regulating separate functions of the cell cycle [37]. CCNA1 also binds to important cell cycle regulators such as Rb family proteins transcription factor E2F-1 and the p21 protein family, and appears to be a downstream player in p53-dependent apoptosis and G2 arrest [38-40]. In cancer, CCNA1 can induce G2 cell cycle arrest, polyplody, apoptosis, and mitotic catastrophe as has been reported for non-small cell lung, ovarian, and renal carcinoma cells [39]. Two primary renal cell carcinomas expressing mutated p53 exhibited reduced or absent expression of CCNA1 relative to the corresponding normal tissue [39]. In prostate cancer, CCNA1 mediates VEGF expression in cooperation with Rb- and androgen-dependent pathways [38]. IHC expression of CCNA1 differs between BPH and cancer as BPH staining is cytoplasmic and pale whereas in cancer the staining is nuclear and related to histologic grade with aggressive tumors showing more intense staining.

In ESCC, we showed that CCNA1 mRNA was elevated in tumors suggesting the gene may be important in the etiology of this neoplasm. While this still may be true, the use of tissue immunostaining as a ‘biological filter’ indicates that the CCNA1 protein appears to be associated with both normal and pathological cell growth and thus may not be a tumor specific change per se. In other words, the apparent up-regulation of CCNA1 mRNA in ESCC could be, at least in part, to the restricted expression in normal epithelium (basal layer only) versus the diffuse expression seen in tumors. Since we microdissected the full thickness of the normal esophageal epithelium, including the differentiated layers without dividing cells, the expression measurements of growth-related genes were biased in favor of the tumor samples. These results highlight that an important next step in identifying and/or validating true growth-related, tumor specific gene expression changes will require direct comparison of microdissected basal esophageal cells (normal growth) versus ESCC (pathological growth) using expression arrays or qRT-PCR analyses [41].

Both the TNFRS19 and TPT1 transcripts were down-regulated in the majority of tumors studied, thus further analysis of these two genes in additional cases of ESCC is indicated. The TNFRS19 gene is located on chromosome 13q12.11-12.3 and is a member of the tumor necrosis receptor superfamily [42, 43]. The protein functions as a cytokine receptor involved with caspase-independent apoptosis and is highly expressed during embryonic development [44]. Receptor ligands are thought to include members of the TRAF family, genes that are important in Drosophila developmental processes via interactions with the JNK and NF-kappaB signaling pathways [45, 46]. Based on its cellular functions and known interactions with tumor necrosis factors, loss of
TNFRS19 in ESCC is a plausible tumor suppressor gene candidate. The TPT1 gene is located on 13q12-14 and its protein product has been reported to be involved in multiple different cellular functions including those related to cellular growth and differentiation [47-53]. At present, the potential role of TPT1 in ESCC formation and development is not clear; however further investigation in more cases is warranted given its chromosomal location and reduced expression in tumors.

CONCLUSION

Quantitative transcript analysis of patient matched normal and tumor samples indicate that the BRCA2 gene does not exhibit loss-of-function in ESCC based on reduced mRNA levels. Additional study in a larger patient population will be required to understand the potential role of the CCNA1, TNFRS19, and TPT1 genes in this cancer type.

ACKNOWLEDGEMENTS

We thank Wusheng Yan and Liang Zhu for discussions about this study, Jeffery Hanson for providing CCR5 primers and probe set sequences, and Keith Killian for DNA extraction advice.

The research was supported by the Intramural Research Programs of the Center for Cancer Research, and the Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

REFERENCES

Interrogation of Chromosome 13q12-14 in Esophageal Squamous Cell Carcinoma

The Open Pathology Journal, 2012, Volume 6


