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

Heidi S. Erickson[1,2], Jaime Rodriguez-Canales[1], Paul S. Albert[3], Kris Yala[4], Sumana Mukherjee[1], Nan Hu[5], Alisa M. Goldstein[5], Rodrigo F. Chuaqui[1], Stephen A. Hewitt[4], Philip R. Taylor[5] and Michael R. Emmert-Buck[*,1]

[1]*Pathogenetics Unit, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA*

[2]*Thoracic/Head & Neck Medical Oncology, Division of Cancer Medicine, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA*

[3]*Biometric Research Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20852, USA*

[4]*Tissue Array Research Program, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA*

[5]*Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA*

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

*Address correspondence to this author at the Pathogenetics Unit, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA; Tel: (301) 496-2912; Fax: (301) 594-7582; E-mail: mbuck@helix.nih.gov

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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 haploinsufficiency, 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 ($\geq$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^{o}C until RNA extraction. A replicate microdissection of all samples was obtained for a second qRT-PCR analysis of *BRCA2* to assess reproducibility. Additional frozen tissue sections were available in five of seventeen cases (case numbers 9, 10, 11, 12, and 16) and were manually microdissected for subsequent DNA extraction.

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^{o} C. Quantitation and qualitation 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].

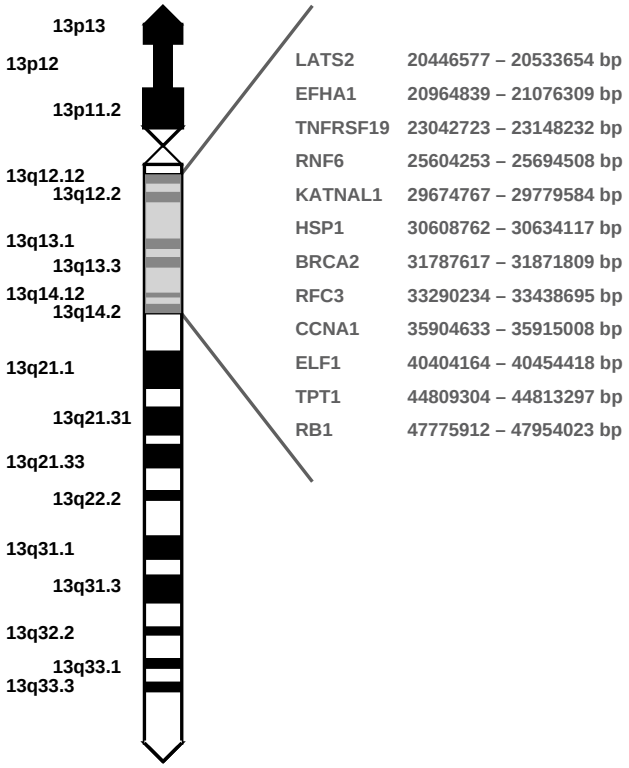

Fig. (1). Schematic of chromosome 13 showing the location of the twelve genes that were analyzed by qRT-PCR in patient-matched normal and tumor samples.

Quantitative Reverse Transcription PCR (qRT-PCR) Gene Expression

RT was conducted on all RNA samples using TaqMan RT Reagents (Applied Biosystems, Inc. (ABI), Foster City, CA, USA; Cat # N808-0234), with random hexamers as the RT primers. cDNA was used immediately for all PCR reactions, with remaining cDNA stored at -80^{o} C.

Fourteen commercially available optimized TaqMan primer/probe sets (Assays-on-Demand, ABI) were used in singleplex qPCR reactions. Beta-actin (*ACTB*) has been shown to be the most stable endogenous control gene between esophageal tumor and normal tissue [23]: therefore, *ACTB* was used as the endogenous control housekeeping gene for normalization. The assays for *LATS2*, *EFHA1*, *TNFRSF19*, *RNF6*, *KATNAL1*, *HSP1*, *BRCA2*, *RFC3*, *CCNA1*, *ELF1*, *TPT1*, and *RB1* are cDNA specific. To assess reproducibility, two *BRCA2* primer/probes sets, each specific to a different exon of the gene (exon 4-5 and exon 13-14, respectively) were used in qPCR analysis of the cDNA samples, and a second qPCR analysis of *BRCA2* exon 13-14

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

Symbol	Gene Name	Map	Category	Gene Group
LATS2	Large tumor suppressor, homolog 2	13q11-q12	Kinase	Protein kinase
EFHA1	EF-hand domain family, member A1	13q12.11	Select calcium binding protein	Calmodulin related protein
TNFRSF19*	tumor necrosis factor receptor superfamily, member 19	13q12.11-q12.3	Receptor	Cytokine receptor
RNF6	ring finger protein (C3H2C3 type) 6	13q12.2	Transcription factor	Transcription cofactor
KATNAL1	katanin p60 subunit A-like 1	13q12.3	Cytoskeletal Protein	Microtubule family cytoskeletal protein
HSP1	heat shock protein1	13q12.3	Chaperone	Hsp 70 family chaperone
BRCA2*	breast cancer 2, early onset	13q12.3	Nucleic acid binding	Damaged DNA-binding protein
RFC3	replication factor C (activator 1) 3, 38kDa	13q12.3-q13	Nucleic acid binding	DNA-directed DNA polymerase
CCNA1**	cyclin A1	13q12.3-q13	Select regulatory molecule	Kinase modulator
ELF1	E74-like factor 1(ets domain transcription factor)	13q14.11	Transcription factor	Other transcription factor
TPT1	tumor protein, translationally-controlled 1	13q12-q14	Cytoskeletal protein	Microtubule family cytoskeletal protein
RB1	retinoblastoma 1	13q14	Transcription factor	Other transcription factor

*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 (C_T) 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 $2^{-\Delta\Delta CT}$ method, with mean biologic fold change calculated directly from mean $\Delta\Delta C_T$ 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.

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 *TNFSFR19*, *BRCA2* and *CCNA1*, and endogenous controls *ACTB* and *CCR5* (C-C chemokine receptor type 5) were employed in the qPCR reactions. All primer/probe sets were DNA specific and commercially available (Assays-on-Demand, ABI), except for *CCR5*, which was designed (*CCR5* Probe 5'-Fam- CTG GGC TCA CTA TGC-MGB; CCR5 FP - TACCTGCTCAA CCTGGCCAT; CCR5 RP - TTCCAAAGTCCCACTGGGC). Controls consisting of human adult normal tissue esophagus DNA (BioChain Institute, Inc. Catalog # D1234106) were positive in all runs, and no-template controls consisting of sterile molecular grade water were negative in all runs.

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. Bonferonni 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/ul; average normal = 16.59 ng/ul) 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 *TPTI*, showed a greater than 1.5 fold decrease in mRNA levels in 10 of 17 and 11 of 17 tumors, respectively (Table **2**). Overall, *TNFRS19* 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 *TNFRS19* 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 $\Delta\Delta C_T$ Values of 13q Region Gene Expression

Case	LATS2	EFHA1	TNFRSF19	RNF6	KATNAL1	HSP1	BRCA2*	RFC3	CCNA1	ELF1	TPT1	RB1
1	2.88	2.29	1.29	1.14	-0.44	-0.07	0.63	-0.12	-4.52	1.60	1.25	-0.04
2	-1.09	-2.15	-0.30	-1.44	-4.30	-3.58	-5.13	-3.79	-4.80	-1.49	-0.20	-2.33
3	-0.56	-0.49	2.55	-0.49	0.71	-0.66	-1.97	-1.32	-11.17	-0.20	1.40	-0.12
4	0.73	-1.51	-0.28	-0.16	-2.00	-3.10	-2.72	-3.14	-11.04	-0.36	1.27	2.74
5	1.54	0.93	3.32	0.90	-0.61	-1.74	-0.34	-0.14	-8.65	0.75	2.71	-0.20
6	-0.58	-0.86	-1.50	-0.23	-3.20	-2.66	-3.59	-3.38	-6.51	-0.55	1.85	-0.85
7	-0.49	-0.62	1.27	-1.00	-2.32	-2.43	-1.75	-2.04	-7.45	-1.95	-0.42	-2.10
8	-0.08	0.58	1.55	-0.06	-1.37	-2.34	-1.41	-1.15	-6.23	-0.23	0.99	-0.36
9	0.60	-0.39	-0.08	-0.42	-1.13	-2.84	-2.16	-2.39	-4.77	-0.68	1.58	-1.41
10	-0.30	-1.34	-3.44	-1.22	-3.09	-3.93	-6.04	-4.34	-18.71	-1.90	-0.15	1.04
11	-1.25	-1.67	2.87	-2.12	-3.19	-2.67	-3.85	-3.25	-9.23	-1.61	-0.45	-2.75
12	0.00	-0.28	3.35	0.93	-1.04	-0.92	-1.44	-1.94	3.24	-0.14	-0.43	-0.91
13	1.73	1.01	3.81	-0.42	-10.76	-2.53	-0.74	-9.84	-7.23	2.11	1.41	3.34
14	0.94	-0.61	-0.15	-1.24	-1.40	-1.89	-2.81	-1.62	0.70	0.84	2.50	4.11
15	-0.20	-0.36	-0.24	-0.06	-1.10	-2.46	-1.52	-0.68	-5.92	-0.52	0.76	-1.18
16	-0.06	-0.34	3.57	-0.18	-0.66	-3.70	-1.96	-2.71	-9.30	-0.42	-3.63	-0.06
17	-0.03	0.45	2.89	2.36	-4.10	-4.20	-2.80	-0.79	-7.67	3.24	2.00	2.87
Mean $\Delta\Delta C_T$	0.22	-0.31	1.21	-0.22	-2.35	-2.45	-2.33	-2.51	-7.02	-0.09	0.73	0.105
S.D.	1.07	1.11	2.06	1.09	2.56	1.14	1.66	2.27	4.79	1.42	1.52	2.05
Mean Fold Change	-1.16	1.24	-2.31	1.16	5.11	5.48	5.02	5.69	129.39	1.06	-1.66	-1.08

*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

Gene	Map Value	Start (bp)	Stop (bp)	Size (bp)	Mean $\Delta\Delta C_T$	S.D.	Mean Fold Change*
LATS2	13q11-q12	20446577	20533654	87077	0.22	1.07	-1.16
EFHA1	13q12.11	20964839	21076309	111470	-0.31	1.11	1.24
TNFRSFR19$^{\downarrow}$	13q12.11-q12.3	23042723	23148232	105509	1.21	2.06	-2.31
RNF6	13q12.2	25604253	25694508	90255	-0.22	1.09	1.16
KATNAL1$^{\uparrow}$	13q12.3	29674767	29779584	104817	-2.35	2.56	5.11
HSP1$^{\uparrow}$	13q12.3	30608762	30634117	25355	-2.45	1.14	5.48
BRCA2$^{\uparrow}$	13q12.3	31787617	31871809	84192	-2.33	1.66	5.02
RFC3$^{\uparrow}$	13q12.3-q13	33290234	33438695	148461	-2.51	2.27	5.69
CCNA1$^{\uparrow *}$	13q12.3-q13	35904633	35915008	10375	-7.02	4.79	129.39
ELF1	13q14.11	40404164	40454418	50254	-0.09	1.42	1.06
TPT1$^{\downarrow}$	13q12-q14	44809304	44813297	3993	0.73	1.52	-1.66
RB1	13q14	47775912	47954023	178111	0.105	2.05	-1.08

Genes without arrow designation had no change in expression of tumor compared to normal (<1.5 fold change).
$^{\downarrow}$Down-regulation of gene expression in tumor tissue (T<N).
$^{\uparrow}$Up-regulation of gene expression in tumor tissue (T>N).
$^{\uparrow *}$4+ (>20 fold) Up-regulation of gene expression in tumor tissue (T>N).
*Mean fold change calculated from average ΔΔCT value of all cases per gene.

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 *TNFRS19*, *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

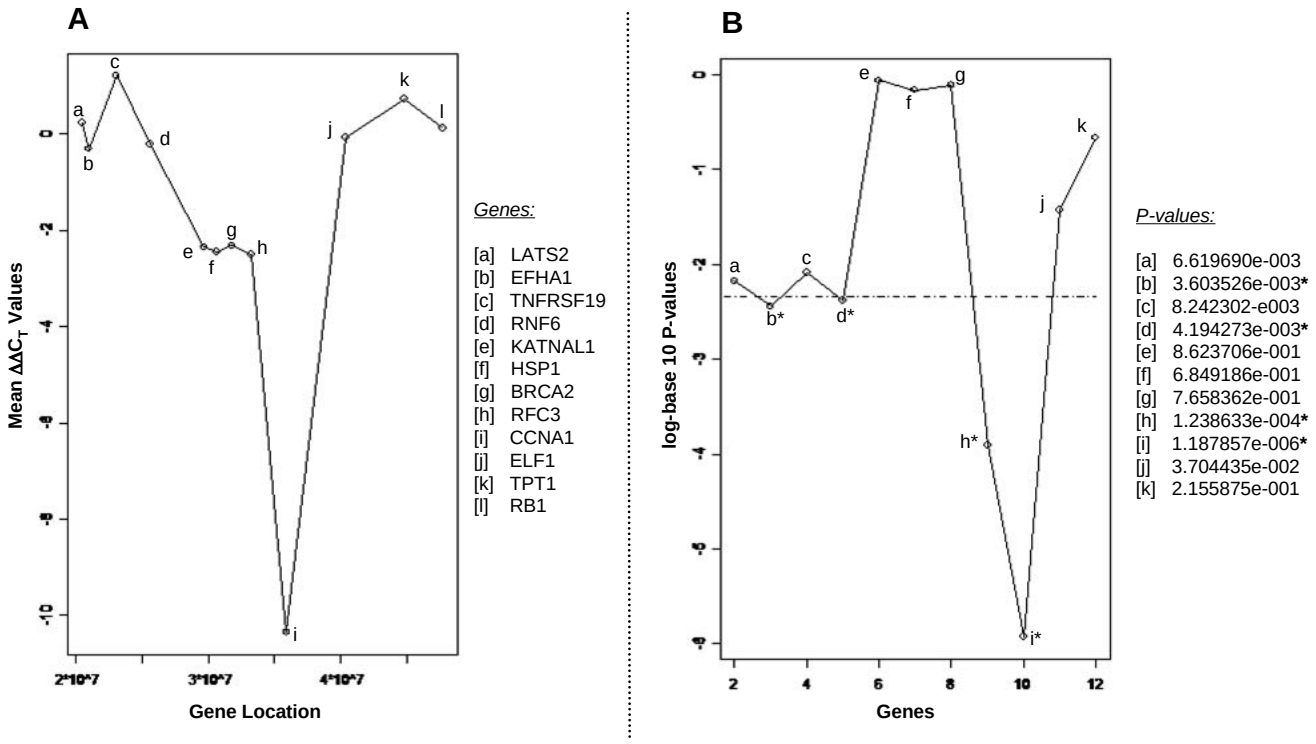

Fig. (2). Mean difference in CT values across each of the genes by spatial location. **A**. Plotted means. Genes analyzed: a = LATS2, b = EFHA1, c = TNFRSF19, d = RNF6, e = KATNAL1, f = HSP1, g = average of 3 BRCA 2 markers, h = RFC3, i = CCNA1, j = ELF1, k = TPT1, l = RB1. **B**. Log-base 10 P-values for two-sided t-tests comparing differences in the CT values across successive genes (located next to each other). Dotted line reflects p-value <0.05/11=0.0045 (accounting for 11 comparisons with a Bonferonni multiple comparisons correction). * = statistical significance representing a shift in mean structure.

making tumor cells susceptible to DNA damaging drugs [30-34].

The *CCNA1* gene is located on chromosome 13q12.3-q13 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, polyploidy, 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 due, 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 at both the genomic and protein levels are 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

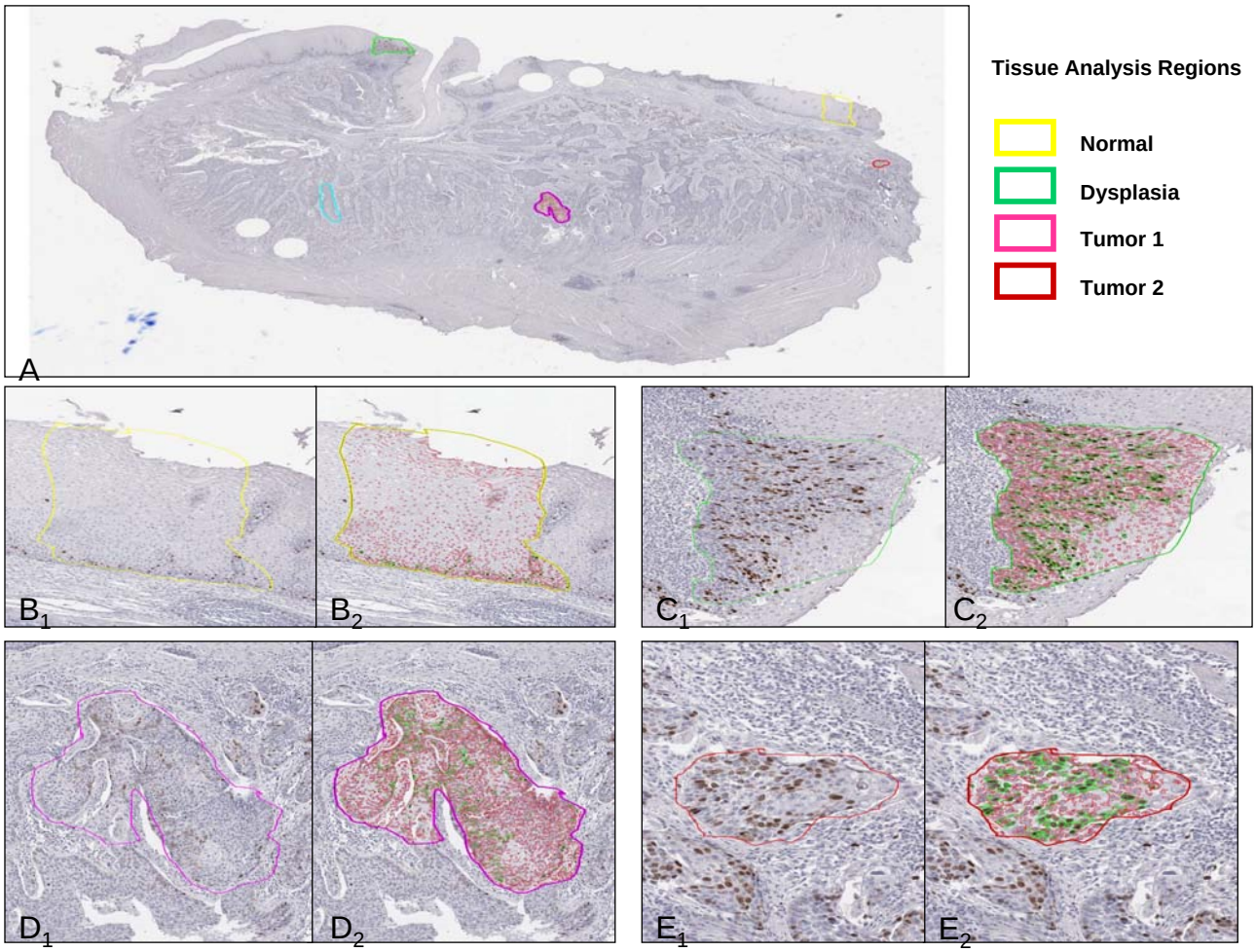

Fig. (3). CCNA1 Immunnohistochemistry (IHC). **A**. Histological map with image analysis areas outlined. **B**. Normal epithelium. **C**. Zone of dysplasia. **D**. Region 1 of tumor. **E**. Region 2 of tumor. $_1$ = Area to be analyzed; $_2$ = Image analysis annotation, green nuclei = positive, pink nuclei = negative. IHC stained, original magnification, x20 (**A**), x200 (**B-E**).

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.

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CONFLICT OF INTEREST

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

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