Gene Expression Profiling Identified High-mobility GroupAT-hook 2 (HMGA2) as Being Frequently Upregulated in Esophageal Squamous Cell Carcinoma

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ABSTRACT

Background: Oesophageal cancer is one of the most deadly malignancies worldwide and oesophageal squamous cell carcinoma (ESCC) is the most frequent type.

Methods: We identified up-regulated genes from gene expression profiles of HKESC-4 cell line, its parental tumor tissues, non-tumoralesophageal epithelia and lymph nodes with metastatic carcinoma using Human Genome U133 plus 2.0 microarray.

Results:Four genes [High-mobility group AT-hook 2 *(HMGA2)*, paternally expressed 10 *(PEG10)*, SH3 and multiple ankyrin repeat domains 2*(SHANK2)* and WNT1 inducible signaling pathway protein 3*(WISP3)*] were selected for further validation with real-time quantitative polymerase chain reaction (PCR) in a panel of ESCC cell lines and clinical specimens. *HMGA2* was found to be overexpressed in the panel of ESCC cell lines tested. By using immunohistochemistry, HMGA2 was found to be up-regulated in 70% of ESCC tissues (21 out of 30 cases).

Conclusion:This study demonstrates successful use of gene microarray to identify and reveal HMGA2 as a novel and consistently overexpressed gene in ESCC cell lines and clinical samples.

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1. Introduction

Esophageal cancer ranks fifth as the most common cause of cancer-related deaths in men worldwide, causing about 400,000 deaths annually[1]. The incident rate is higher in Southern and Eastern Africa and Eastern Asia when compared to Western and Middle Africa and Central America[1]. This cancer comprises two major types, namely esophageal squamous cell carcinoma (ESCC) and adenocarcinoma, the former is more common and contributes to about 90% of cases in high-risk regions [1]. Patients with ESCC usually have poor prognosis largely because of late diagnosis of the disease[2]. Despite advances in surgical techniques combined with various treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate remains at20-30%[3]. To alleviate this clinical situation, the development of new treatment modalities, diagnostic technologies and preventative measures is required, which cannot be accomplished without understanding the underlying mechanisms of esophageal carcinogenesis. The development of gene microarray technology allows comprehensive comparison of gene expression profiles in various pathophysiological processes, such as enabling the comparison of gene profiles between cancer and normal conditions. In the present study, we took advantage of this technology to identify differentially expressed genes in cancerous and non-cancerous conditions in esophagus, followed by further validation for the involvement of these genes in ESCC.

2. Materials and Methods

*HKESC-4 cell line and clinical specimens for microarray.*HKESC-4, a human ESCC cell line of Chinese origin, was established previously in our laboratory and the culture conditionsfor this cell line were as described elsewhere [4]. Cultured HKESC-4 cells were harvested at 80% confluency at passage 30 for extracting RNA. Parental tumoral tissue (T), from which HKESC-4 cell line was derived, and its corresponding non-tumoral epithelium (N) and lymph node containing metastatic carcinoma (LN) were isolated during esophagectomy and snap-frozen until their use for RNA extraction. The extracted RNA of the cell line (HKESC-4) and clinical specimens (T, N and LN) was subjected to gene microarray. Consent regarding the use of clinical specimens for this study was obtained from Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB).

Clinical tissues and ESCC cell lines for quantitative real time polymerase chain reaction (qPCR). In addition to the clinical specimens for gene microarray as mentioned above, three non-tumoral tissues were obtained from esophageal epithelium at least 5 cm away from the tumorfrom other patients during surgical resection (Non-T). Apart from HKESC-4 cell line, HKESC-1, HKESC-2, HKESC-3 and SLMT-1 cell lines were included and used as described elsewhere [4-6]. These cells were cultured in minimal essential medium (Invitrogen,

Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin. Cell cultures were maintained in a humidified incubator at 37°C containing 5% carbon dioxide (CO_2). Monolayer cells at 80% confluency were harvested and used for RNA extraction.

*RNA extraction for qPCR.*RNA extraction was performed as described [7]. In brief, 1 ml TRIzol reagent (Invitrogen) was used to lyse tissues or cells for RNA extraction. Chloroform was used for phase separation. After centrifugation, the upper aqueous phase with RNA was collected and transferred to RNase-free tubes containing 0.5 ml isopropyl alcohol for RNA precipitation. RNA pellets were then washed with 75% RNase-free ethanol. Finally, RNA pellets were dissolved in RNase-free water. DNase I digestion was performed before the concentration of RNA was determined by measuring its absorbance at 260 nm and A_{260}:A_{280} ratio

*Gene microarray.*RNA of HKESC-4 cells and clinical specimens were subjected to gene expression profiling using GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). This GeneChip enables the analysis of over 47,000 human transcripts and variants. The whole procedure of RNA quality control, microarray labeling,GeneChip hybridization and data acquisition was performed at the Genome Research Centre, The University of Hong Kong, Hong Kong, PRC under standardized condition. The statistical analysis to identify differentially expressed genes was performed using MicroArray Suite software (Affymetrix).

*Synthesis of complementary DNA (cDNA).*RNA was reverse-transcribed into cDNAusing High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Invitrogen), according to the manufacturer's protocol. Briefly, DNase-treated RNA was diluted with RNase-free water to reach a final concentration of 250ng in 10µl. Diluted RNA was then mixed with 2µl 10X RT buffer, 0.8 µl 25X dNTP Mix (100 mM), 2µl10X RT Random Primers, 1µlMultiScribe Reverse Transcriptase (50 U/µl), 1 µlRNase Inhibitor and 3.2 µlnuclease-free water. The reaction was then incubated at 25°C for 10 minutes, followed by 37°C for 2 hours and 85°C for 5 seconds to inactivate the activities ofthe reverse transcriptase and to completely denature the template.

qPCR. The procedure for qPCR was followed as described elsewhere [8, 9]. In brief,qPCR was performed usingcDNA of each sample, gene-specific primers (Table 1) and Platinum SYBR Green qPCRSuperMix-UDG (Invitrogen), according to the protocol from the manufacturer. The reactions were run for 50 cycles at 94°C for 10 minutes, 95°C for 90 seconds and 72°C for 90 seconds in an ABI PRISM 7700 Sequence Detector (Applied Biosystems).Cycle threshold (C_T)values of each reaction were obtained using Sequence Detection System (SDS) Software Version 1.9.1. For each reaction, the expression of each

gene was normalized against the expression of the housekeeping gene □-actin. The relative expression of each gene was calculated based on a comparative C_T equation and is presented as the value of relative intensity.

*Statistical analysis for qPCR data.*Statistical analysis was performed as described elsewhere [10]. One-way ANOVA followed by Duncan's multiple range testwas used to determine statistical significance. Each sample was run in triplicates. The relative value is presented as the mean ± standard error of the mean (S.E.M.). *p*-Values less than 0.05 were considered statistically significant. Statistical analysis was performed with Statistical Package for the Social Sciences version 17 (IBM, New York, USA).

*Immunohistochemistry.*Immunohistochemistry was performed using the avidin-biotin method[11, 12].Five-micrometer paraffin sections were prepared on gelatin-coated glass slides. Sections were preheated at 60°C for 20 minutes, deparaffinized in xylene and rehydrated through graded alcohol. Antigen retrieval was carried out by heating the sections in 0.2M citrate buffer (pH6) in a microwave oven at 95°Cfor 5 minutes. After cooling for 30 minutes, the sections were treated with 0.3% hydrogen peroxide at room temperature for 30 minutes to block endogenous peroxidase activity. Non-specific binding sites on sections were blocked with 1XTBS with 2% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA) and 2% normal goat serum (Invitrogen) at room temperature for 30 minutes. HMGA2-specific rabbit polyclonal antibody (1:100)(Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied to the sections and the reaction was incubated at room temperature for 30 minutes. After washing three times with 1X TBS for 5 minutes each, the sections were incubated with biotinylatedanti-rabbit secondary antibody (EnVision Systems;Dako, Glostrup, Denmark) at room temperature for 30 minutes. To visualize the signals, the sections were washed and stained with avidin-biotin complex and 3,3'-diaminobenzene (DAB) at room temperature.Lastly, the sections were counterstained with hematoxylin. The expressions of HMGA2 were evaluated by Dr. AK Lam, a qualified pathologist, under light microscope.

3. Results

*Identification of differentially expressed genes using gene microarray.*Gene microarray analysis revealed 3,081 genes, 4,027 genes and 3,590 genes having more than 2-fold induction in T, LN and HKESC-4 cells when compared to N. For down-regulated genes with less than 2-fold difference, 4,808 genes, 6,052 genes and 5,846 genes were found in T, LN and HKESC-4 cells in comparison with N. For those up-regulated genes in T, LN and HKESC-4, 43 of them had more than 10-fold induction (Table 2). Among them, 34 genes (*CAMK2A*, *CART1*, *CCNA1*, *COCH*, *FLJ33516*, *FMN2*,*FOXD1, FOXG1B, GPR*, *HMGA2*,*HOXC10*, *HOXC11*, *HTR2C*, *IMP-3*, *LOC163782*,*MAGEB2*, *MFAP2*, *MGC17986*,

MGC27005, *NBEA*, *NKX2-2, PCDHB5*, *PEG10*, *PFN2*, *POPDC3*, *PPFIA1*, *PRAME*, *SAGE1*, *SHANK2*, *SIX1*, *SLCO1B3*, *SYT1, TP53TG3* and *WISP3*) were first identified to be overexpressed in ESCC, while 13 of them (*DKK1*,*EGFR, EMS1*, *GAL*, *HCG4*, *LAMC2*,*MAGEA1, MAGEA4,MAGEA11*, *MMP13*, *PTHLH*,*ZIC* and *ZNF595*)have previously been reported to be overexpressed in ESCC (Table 3). Among the 34 newly identified genes, *HMGA2*, *PEG10*, *SHANK2* and *WISP3*were further selected for validation due to their potential involvement in tumorigenesis based on literature search.

*Gene expression of HMGA2.*High expression of *HMGA2* in N, T, LN and HKESC-4 observed in gene microarray was confirmed using qPCR, such that the relative intensitiesof *HMGA2* expression in N, T, LN and HKESC-4 obtained using these two methods were comparable (fold change by gene microarray: 1, 31, 63 and 18*versus*1, 26, 49 and 33 by qPCR). Moreover, increased gene expression of *HMGA2*was also detected in four ESCC cell lines (HKESC-1, HKESC-2, HKESC-3 and SLMT-1). Significantly higher gene expression on average of 20-fold of *HMGA2*was noted in ESCC cell lines when compared to N and Non-T (Figure 1A).

*Gene expression of PEG10.*An over-expression of *PEG10*in ESCC observed in gene microarray was confirmed using qPCR. Therelative intensities of *PEG10* in N, T, LN and HKESC-4 cells were 1, 20, 28 and 28 by gene microarray, while their relative intensities were 1, 45, 148 and 269 byqPCR, respectively. However, no significant difference in the average gene expression of *PEG10* was detected in tested ESCC cell lines when compared to N and Non-T, despite their having an average of 100-fold induction in gene expression of *PEG10* (Figure 1B).

Gene expression of SHANK2

High gene expression of *SHANK2* in ESCC observed in gene microarray was confirmed using qPCR. The relative intensities of *SHANK2* in N, T, LN and HKESC-4 cells were 1, 30, 20 and 20by gene microarray and 1, 21, 11 and 19 byqPCR, respectively. No significant difference in the gene expression of *SHANK2* was detected in ESCC cells when compared to N (Figure 1C).

*Gene expression of WISP3.*High gene expression of *WISP3* in ESCC detected using gene microarray was confirmed using qPCR. The relative intensities of gene expression of *WISP3* in N, T, LN and HKESC-4 cells were 1, 37, 53 and 26by gene microarray and 1, 152, 117 and 117byqPCR, respectively. A significantincrease in the gene expression of *WISP3* was detected in HKESC-2 cells. An average of 100-foldhigher gene expression of *WISP3* was found when compared to N and Non-T (Figure 1D).

*Protein expression of HMGA2 in ESCC tissues.*Immunohistochemical data show the localization of HMGA2 in the nuclei of the ESCC tissues(Figure 2). Overexpression of HMGA2 was found in 70% ESCC tissues (21 out of 30 cases) when the expression of HMGA2 was examined in 30 pairs of tumoral tissues and adjacent non-tumoral tissues. No detectable level of HMGA2 expression was observed in the non-tumoral tissues(data not shown).

4. Discussion

In the present study, to our knowledge, the gene microarray and real-time qPCR analysis showed the presence of *HGMA2*mRNA expression in human esophageal tissues for the first time. *HGMA2* mRNA overexpression was detected in ESCC cell lines compared with the corresponding morphologically non-tumoralesophageal epithelial tissues. The current findings showed that overexpression of *HMGA2* gene appearsto be a consistent feature in ESCC. The protein expression of HMGA2 was further validated in ESCC specimens by immunohistochemistry. Liu *et al.*have shown an elevated level of HMGA2 protein in ESCC tissues by comparing 150 pairs oftumor and adjacent non-tumor tissues in patients[13], for which this data are in line with our findings reported here.The immunostaining analysis showed that HMGA2 protein expression was localized in the nuclei of the ESCC cells. The majority of ESCC cases (21/30, 70%) were found to have significantly enhanced expression of HMGA2 compared with morphologically normal esophageal epithelium.

HMGA2 belongs to the HMGA family, which also contains two other members *HMGA1A* and *HMGA1B*. HMGA protein family members are small nuclear proteins. A prominent feature of the HMGA family is the three DNA-binding domains termed AT-hooks at the *N*-terminal region that bind the minor groove of AT-rich DNA sequences. These proteins play key roles in chromatin architecture and gene control by serving as generalized chromatin effectors, either enhancing or suppressing the ability oftranscriptional factors in the process of transcriptional regulation. HGMA2 expression was found to be restricted during embryogenesis, whereas it is absent or has low expression in normal adult tissues[14]. However, overexpression of HGMA2 has been reported in various types ofhuman cancer including of the pituitary [15], oral cavity [16], lung [17], breast [18], pancreas [19], and nerves[20]. In addition, HMGA2 protein was reported to be ectopically expressed at the invasive front of oral carcinomas and had a significant impact on tumor progression and patient survival [16]. Similarly, HMGA proteins were found to be expressed in lung carcinomas and their expressions were inversely associated with survival, providing a potentially useful marker for diagnosis and prognosis of lung cancer [21].

Overexpression of *HMGA2* gene leads to pituitary adenomas in mice. The mechanism has been described by Fedele*et al*.[22]. HMGA2 binds to the pRB A/B pocket domain, while it does not compete with the E2F1 protein. Conversely, E2F1 activation by HMGA2 occurs by displacing HDAC1 from the pRB/ E2F1 complex, resulting in enhanced acetylation of both E2F1 and DNA-associating histones, thereby promoting E2F1 activation [22]. It is well-known that pRB controls cell cycle progression through its interaction with the E2F family of transcription factors, whose activity is crucial for the expression of several genes required for cells to enter the S phase of the cell cycle[23]. By repressing E2F1 activity, pRB protein prevents cell from progressing beyond the G1 phase of the cell cycle. If the repression of E2F1 is relieved by phosphorylation or viral transformation of pRB[24, 25], resulting in the release of E2F1, the transcription of its target genes is activated[26]. This allows cells to progress toward S phase. The overexpressions of pRB[27] and E2F1[28] were also found in ESCC specimens.These findings are consistent with the mechanism in pituitary cancer described by Fedele*et al*.. This suggests that the pRB/E2F1 pathway involving *HMGA2* may also play a critical role in the pathogenesis of ESCC.

In summary, the gene microarray results show a comprehensive picture of the differential gene expression in ESCC.Thirty novel overexpressed genes were revealed in this study. The real-time qPCR results confirmed that *HMGA2* was up-regulated in all the ESCC cell lines. In addition, the protein expression of HMGA2 demonstrated a significantly higher incidence of overexpression in primary ESCCs than morphologically non-tumoralesophageal epithelium tissue. For the first time, the present findings showed that *HMGA2* was overexpressed in ESCC, and suggest that the activation of HMGA2 might be important in the pathogenesis of ESCC.

Table 1. Primer sequences of studied genes.

Gene	DNA sequences
HMGA2	5'-CAGCAGCAAGAACCAACC-3' 5'-CAGTTTCCTCCTGAGCAG-3'
PEG10	5'-GGGTCTGTCATCGACTAC-3' 5'-CTCGGTTGGATCTACCTG-3'
SHANK2	purchased from SuperArray Bioscience Corporation (Frederick, MD, USA)
WISP3	5'-CAGCAGCTTTCAACAAGCTACA-3' 5'-TTCCCATCCCACATGTTCTG-3'
β-Actin	5'-GCTCGTCGTCGACAACGGCTC-3' 5'-CAAACATGATCTGGGTCATCTTCTC-3'

Table 2. Genes with >10-fold higher expression in esophageal squamous cell carcinoma tissues and cells studied

Gene Symbol	Position	Chromosome location	Relative intensity*		
			Tumor	Lymph node metastasis	HKESC-4 cells
CAMK2A	229163_at	1p36.13	24.08	17.26	20.94
CAMK2A	218309_at	1p36.13	22.7	17.76	23.08
CAMK2A	228302_x_at	1p36.13	17.06	11.62	28.18
CART1	206837_at	12q21.3-q22	16.01	15.6	14.43
CCNA1	205899_at	13q12.3-q13	50.35	58.86	48.36
COCH	205229_s_at	14q12-q13	14.14	13.1	11.59
DKK1	204602_at	10q11.2	64.93	96.28	344.8
EGFR	201984_s_at	7p12	22.85	31.67	30.82
EMS1	214073_at	11q13	17.43	17.63	21.22
FLJ33516	229160_at	Xq22.3	18.6	53.55	32.52
FMN2	223618_at	1q43	16.56	22.98	22.86
FMN2	1555471_a_at	1q43	11.93	14.46	17.5
FOXD1	206307_s_at	5q12-q13	15.44	20.53	13.95
FOXG1B	206018_at	14q12-q13	18.82	24.46	30.44
GAL	214240_at	11q13.1	41.56	46.58	41.16
GPR	227846_at	15q14	12.91	11.44	13.84
HCG4	206685_at	6p21.3	11.44	21.18	7.994
HMGA2	208025_s_at	12q15	31.22	62.65	17.94
HOXC10	218959_at	12q13.3	22.96	22.56	31.13
HOXD10	229400_at	2q31.1	13.15	15.05	21.27
HOXD11	214604_at	2q31.1	20.12	26.2	23.37
HTR2C	207307_at	Xq24	21.95	18.02	26.57
IMP-3	203820_s_at	7p11	21.56	35.95	30.82
LAMC2	202267_at	1q25-q31	21.33	28.72	11.3
LOC163782	229125_at	1p32.1	14.43	11.71	39.89
MAGEA1	207325_x_at	Xq28	89.08	192.8	129.5
MAGEA11	210503_at	Xq28	36.73	43.59	25.86
MAGEA4	214254_at	Xq28	30.04	47.19	28.42
MAGEB2	206218_at	Xp21.3	101.3	111.9	113.1
MFAP2	203417_at	1p36.1-p35	47.36	25.34	14.93
MGC17986	1552946_at	19q13.33	19.44	21.97	18.82
MGC27005	1567912_s_at	---	345.1	662	441.5
MGC27005	235700_at	Xq26.3	227.8	410.3	305.3
MMP13	205959_at	11q22.3	206.5	82.06	46.82
NBEA	239010_at	---	24.72	19.12	21.34
NKX2-2	206915_at	20pter-q11.23	20.36	20.32	19.78
PCDHB5	223629_at	5q31	13.68	19.72	25.47
PEG10	212094_at	7q21	19.58	27.79	27.8
PFN2	204992_s_at	3q25.1-q25.2	14.39	20.15	17.83
POPDC3	219926_at	6q21	20.75	26.54	28.86
PPFIA1	210236_at	11q13.2	14.19	17.12	15.62
PRAME	204086_at	22q11.22	11.47	17.7	13.24
PTHLH	1556773_at	12p12.1-p11.2	42.7	120.4	67.42
PTHLH	206300_s_at	12p12.1-p11.2	41.34	76.86	48.18
PTHLH	211756_at	12p12.1-p11.2	35.64	76	34.72
SAGE1	220793_at	Xq26	15.89	20.5	56.04
SHANK2	213308_at	11q13.2	29.9	19.83	19.99
SHANK2	213307_at	11q13.2	19.41	14.38	15.04
SIX1	228347_at	14q23.1	20.37	23.38	19.25
SLCO1B3	206354_at	12p12	13.48	27.82	30.46
SYT1	203999_at	12cen-q21	21.44	20.41	21.4
TP53TG3	220167_s_at	16p13	16.8	12.91	10.12

WISP3	210861_s_at	6q22-q23	36.77	52.97	26.4
ZIC1	206373_at	3q24	61.6	70	97.5
ZNF595	227952_at	---	20.42	39.94	31.02

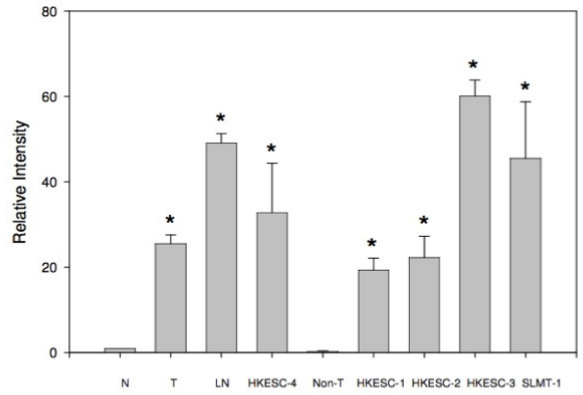
* Relative to morphologically normal esophageal epithelium

Table 3. Genes found to have overexpression in ESCC in previous studies.

Gene Symbol	Description	Reference
DKK1	Overexpression of *DKK1* gene in the distal squamous esophageal mucosa in patients with esophagitis	[29]
EGFR	Overexpression of *EGFR* in ESCC and its correlation with depth of tumorinvasion	[30]
EMS1	Association of amplification and overexpression of *EMS1* with lymph node metastasis in ESCC	[31]
GAL	Distribution of *galanin*(GAL) immunoreactive nerve bundles and scattered nerve fibres in esophageal carcinoma	[32]
HCG	High expression of *HCG* expression in patients with lymph node metastasis and its correlation with infiltration and metastasis	[33]
LAMC2	Co-expression of *LN-5 gamma2* (*LAMC2*) and *EGFR* is closely related to the progression and poor prognosis of ESCC	[34]
PTHLH	High level of serum parathyroid hormone-related protein (*PTHLH*) in esophageal carcinoma	[35]
MAGE-A, MMP13, ZNF595	High expression of *MAGE-A*, *MMP13*and zinc finger proteinsin ESCC	[36]

Figure 1.

A

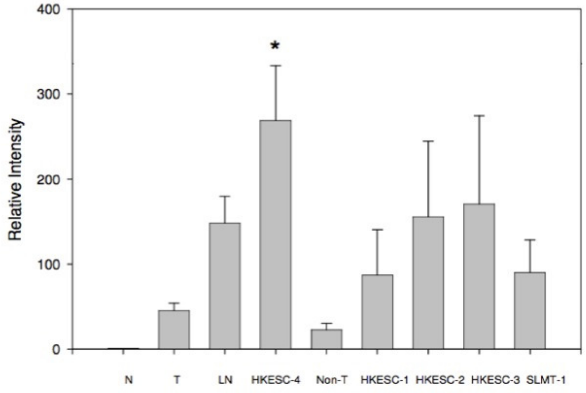


The relative intensity of HMGA2 gene expression:

N	T	LN	HKESC-4	Non-T	HKESC-1	HKESC-2	HKESC-3	SLMT-1
1.00 ± 0.00	25.50 ± 2.06 *	49.06 ± 2.25 *	32.77 ± 11.58 *	0.27 ± 0.17	19.35 ± 4.99 *	22.28 ± 3.78 *	60.08 ± 13.23 *	45.51 ± 4.21 *

Note: *: significant difference from N ($p<0.05$).

B

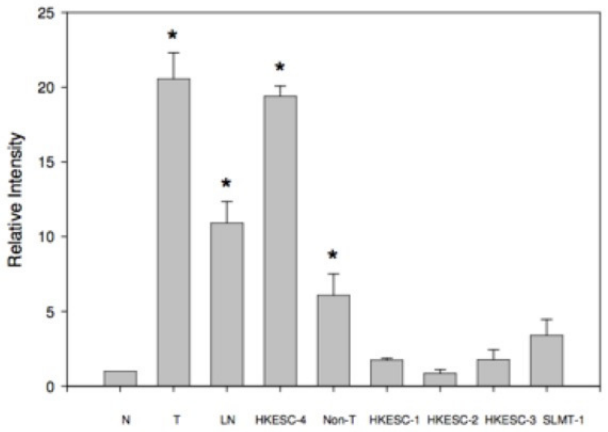


The relative intensity of PEG10 gene expression:

N	T	LN	HKESC-4	Non-T	HKESC-1	HKESC-2	HKESC-3	SLMT-1
1.00 ± 0.00	45.25 ± 8.85	148.14 ± 31.54	269.01 ± 64.30 *	22.52 ± 7.83	86.82 ± 53.82	155.52 ± 89.04	170.43 ± 103.88	90.05 ± 38.18

Note: *: significant difference from N ($p<0.05$).

C

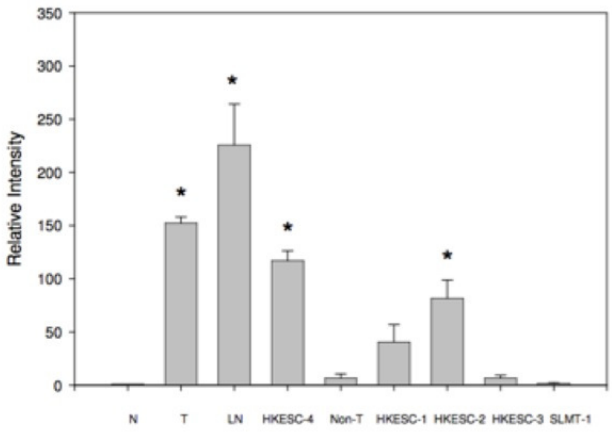


The relative intensity of Shank2 gene expression:

N	T	LN	HKESC-4	Non-T	HKESC-1	HKESC-2	HKESC-3	SLMT-1
1.00 ± 0.00	20.55 ± 3.04 *	10.93 ± 2.47 *	19.40 ± 1.16 *	6.08 ± 2.48 *	1.75 ± 0.20	0.85 ± 0.44	1.76 ± 1.17	3.40 ± 1.83

Note: *: significant difference from N ($p<0.05$).

D



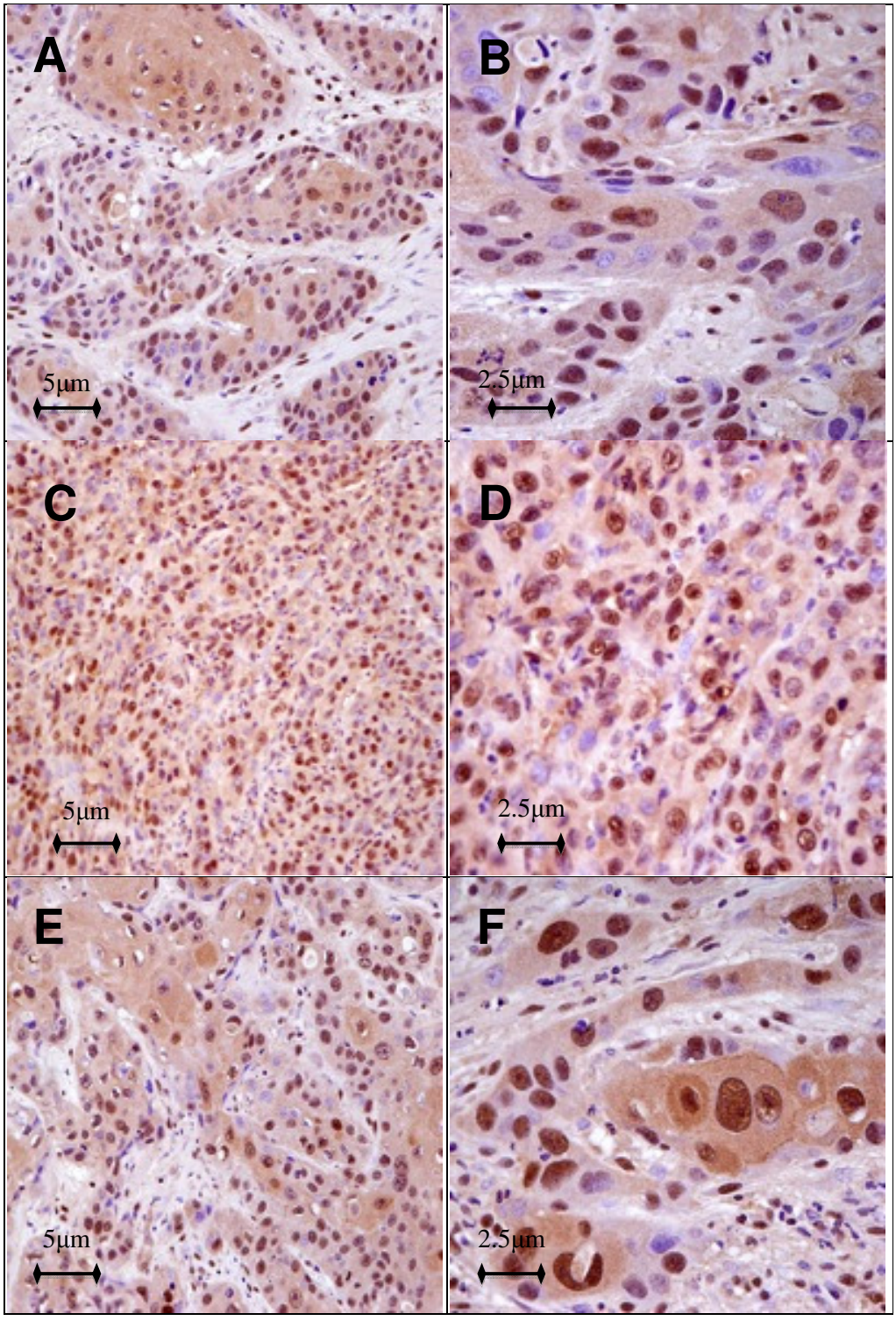
The relative intensity of WISP3 gene expression:

N	T	LN	HKESC-4	Non-T	HKESC-1	HKESC-2	HKESC-3	SLMT-1
1.00 ± 0.00	152.42 ± 5.49 *	116.90 ± 38.61 *	116.90 ± 9.45 *	6.51 ± 4.00	40.50 ± 16.47	81.70 ± 17.10 *	6.64 ± 2.64	1.57 ± 0.78

Note: *: significant difference from N ($p<0.05$).

N	Morphologically normal esophageal epithelium collected from the same patient from which the HKESC-4 was derived.
T	Tumor esophageal epithelium collected from the same patient from which the HKESC-4 was derived.
LN	Lymph node metastasis of ESCC collected form the same patient from which HKESC-4 was derived.
Non-T	Another three non-tumoral esophageal tissues from esophageal cancer patients.

Figure 2.



References

1. Jemal A, Bray F, Center MM*, et al* (2011) Global cancer statistics. CA: a cancer journal for clinicians 61: 69-90
2. Shimada H, Nabeya Y, Okazumi S*, et al* (2003) Prediction of survival with squamous cell carcinoma antigen in patients with resectable esophageal squamous cell carcinoma. Surgery 133: 486-494
3. Isono K, Sato H and Nakayama K (1991) Results of a nationwide study on the three-field lymph node dissection of esophageal cancer. Oncology 48: 411-420
4. Cheung LC, Tang JC, Lee PY*, et al* (2007) Establishment and characterization of a new xenograft-derived human esophageal squamous cell carcinoma cell line HKESC-4 of Chinese origin. Cancer Genet Cytogenet 178: 17-25
5. Hu Y, Lam KY, Wan TS*, et al* (2000) Establishment and characterization of HKESC-1, a new cancer cell line from human esophageal squamous cell carcinoma. Cancer genetics and cytogenetics 118: 112-120
6. Tang JC, Wan TS, Wong N*, et al* (2001) Establishment and characterization of a new xenograft-derived human esophageal squamous cell carcinoma cell line SLMT-1 of Chinese origin. Cancer genetics and cytogenetics 124: 36-41
7. Hui MK, Lai KK, Chan KW*, et al* (2012) Clinical correlation of nuclear survivin in esophageal squamous cell carcinoma. Medical oncology
8. Lee NP, Leung KW, Cheung N*, et al* (2008) Comparative proteomic analysis of mouse livers from embryo to adult reveals an association with progression of hepatocellular carcinoma. Proteomics 8: 2136-2149
9. Lee NP, Tsang FH, Shek FH*, et al* (2010) Prognostic significance and therapeutic potential of eukaryotic translation initiation factor 5A (eIF5A) in hepatocellular carcinoma. International journal of cancer. Journal international du cancer 127: 968-976
10. Hui MK, Lai KK, Chan KW*, et al* (2011) Prognostic significance of phosphorylated RON in esophageal squamous cell carcinoma. Medical oncology
11. Liu LX, Lee NP, Chan VW*, et al* (2009) Targeting cadherin-17 inactivates Wnt signaling and inhibits tumor growth in liver carcinoma. Hepatology 50: 1453-1463
12. Chung Y, Lam AK, Luk JM*, et al* (2007) Altered E-cadherin expression and p120 catenin localization in esophageal squamous cell carcinoma. Annals of surgical oncology 14: 3260-3267
13. Liu Q, Lv GD, Qin X*, et al* (2012) Role of microRNA let-7 and effect to HMGA2 in esophageal squamous cell carcinoma. Mol Biol Rep 39: 1239-1246
14. Zhou X, Benson KF, Ashar HR*, et al* (1995) Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. Nature 376: 771-774
15. Finelli P, Pierantoni GM, Giardino D*, et al* (2002) The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas. Cancer Res 62: 2398-2405
16. Miyazawa J, Mitoro A, Kawashiri S*, et al* (2004) Expression of mesenchyme-specific gene HMGA2 in squamous cell carcinomas of the oral cavity. Cancer Res 64: 2024-2029
17. Wikman H, Kettunen E, Seppanen JK*, et al* (2002) Identification of differentially expressed genes in pulmonary adenocarcinoma by using cDNA array. Oncogene 21: 5804-5813
18. Langelotz C, Schmid P, Jakob C*, et al* (2003) Expression of high-mobility-group-protein HMGI-C mRNA in the peripheral blood is an independent poor prognostic indicator for survival in metastatic breast cancer. British journal of cancer 88: 1406-1410
19. Abe N, Watanabe T, Suzuki Y*, et al* (2003) An increased high-mobility group A2 expression level is associated with malignant phenotype in pancreatic exocrine tissue. British journal of cancer 89: 2104-2109
20. Giannini G, Di Marcotullio L, Ristori E*, et al* (1999) HMGI(Y) and HMGI-C genes are expressed in neuroblastoma cell lines and tumors and affect retinoic acid responsiveness. Cancer research 59: 2484-2492
21. Sarhadi VK, Wikman H, Salmenkivi K*, et al* (2006) Increased expression of high mobility group A proteins in lung cancer. The Journal of pathology 209: 206-212
22. Fedele M, Pierantoni GM, Visone R*, et al* (2006) Critical role of the HMGA2 gene in pituitary adenomas. Cell cycle 5: 2045-2048
23. Muller H, Bracken AP, Vernell R*, et al* (2001) E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes & development 15: 267-285
24. Brown VD and Gallie BL (2002) The B-domain lysine patch of pRB is required for binding to large T antigen and release of E2F by phosphorylation. Molecular and cellular biology 22: 1390-1401

25. Seville LL, Shah N, Westwell AD*, et al* (2005) Modulation of pRB/E2F functions in the regulation of cell cycle and in cancer. Current cancer drug targets 5: 159-170
26. Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81: 323-330
27. Kawakubo H, Ozawa S, Ando N*, et al* (2005) Alterations of p53, cyclin D1 and pRB expression in the carcinogenesis of esophageal squamous cell carcinoma. Oncology reports 14: 1453-1459
28. Ebihara Y, Miyamoto M, Shichinohe T*, et al* (2004) Over-expression of E2F-1 in esophageal squamous cell carcinoma correlates with tumor progression. Diseases of the esophagus : official journal of the International Society for Diseases of the Esophagus / I.S.D.E 17: 150-154
29. Yamabuki T, Takano A, Hayama S*, et al* (2007) Dikkopf-1 as a novel serologic and prognostic biomarker for lung and esophageal carcinomas. Cancer research 67: 2517-2525
30. Hanawa M, Suzuki S, Dobashi Y*, et al* (2006) EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. International journal of cancer. Journal international du cancer 118: 1173-1180
31. Luo ML, Shen XM, Zhang Y*, et al* (2006) Amplification and overexpression of CTTN (EMS1) contribute to the metastasis of esophageal squamous cell carcinoma by promoting cell migration and anoikis resistance. Cancer research 66: 11690-11699
32. Sugimoto T, Seki N, Shimizu S*, et al* (2009) The galanin signaling cascade is a candidate pathway regulating oncogenesis in human squamous cell carcinoma. Genes, chromosomes & cancer 48: 132-142
33. Li DM, Li SS, Zhang YH*, et al* (2005) Expression of human chorionic gonadotropin, CD44v6 and CD44v4/5 in esophageal squamous cell carcinoma. World journal of gastroenterology : WJG 11: 7401-7404
34. Shen XM, Wu YP, Feng YB*, et al* (2007) Interaction of MT1-MMP and laminin-5gamma2 chain correlates with metastasis and invasiveness in human esophageal squamous cell carcinoma. Clin Exp Metastasis 24: 541-550
35. Watanabe HA, Matsushita H, Matsui H*, et al* (1999) Esophageal carcinoma with high serum parathyroid hormone-related protein (PTHrP) level. Journal of gastroenterology 34: 510-515
36. Du XL, Hu H, Lin DC*, et al* (2007) Proteomic profiling of proteins dysregulted in Chinese esophageal squamous cell carcinoma. Journal of molecular medicine 85: 863-875

Figure legends

Figure 1.Gene expressions of *HMGA2* (A), *PEG10* (B), *SHANK2* (C) and *WISP3* (D) relative to β-actin have been shown in clinical tissues and ESCC cell lines. The value of N is arbitrarily set to 1 for comparison.

Figure 2.Immunohistochemicalstaining of HMGA2 in ESCC tissues. Original magnification, 200X(A, C and E); 400X (B, D and F).