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

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56 **Abstract.** Background: Esophageal cancer is one of the most deadly malignancies worldwide and esophageal
57 squamous cell carcinoma (ESCC) is the most frequent type. Methods: We identified up-regulated genes from
58 gene expression profiles of HKESC-4 cell line, its parental tumor tissues, non-tumoral esophageal epithelia and
59 lymph nodes with metastatic carcinoma using Human Genome U133 plus 2.0 microarray. Results: Four genes
60 [High-mobility group AT-hook 2 *(HMGA2)*, paternally expressed 10 *(PEG10)*, SH3 and multiple ankyrin repeat
61 domains 2 *(SHANK2)* and WNT1 inducible signaling pathway protein 3 *(WISP3)*] were selected for further
62 validation with real-time quantitative polymerase chain reaction (PCR) in a panel of ESCC cell lines and clinical
63 specimens. *HMGA2* was found to be overexpressed in the panel of ESCC cell lines tested. By using
64 immunohistochemistry, HMGA2 was found to be up-regulated in 70% of ESCC tissues (21 out of 30 cases).
65 Conclusion: This study demonstrates successful use of gene microarray to identify and reveal HMGA2 as a
66 novel and consistently overexpressed gene in ESCC cell lines and clinical samples.
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69 **Key Words:** Esophageal cancer, microarray, HMGA2, PEG10, SHANK2, WISP3.
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71
72 **Introduction**

73 Esophageal cancer ranks fifth as the most common cause of cancer-related deaths in men worldwide, causing
74 about 400,000 deaths annually [1]. The incident rate is higher in Southern and Eastern Africa and Eastern Asia
75 when compared to Western and Middle Africa and Central America [1]. This cancer comprises two major types,
76 namely esophageal squamous cell carcinoma (ESCC) and adenocarcinoma, the former is more common and
77 contributes to about 90% of cases in high-risk regions [1]. Patients with ESCC usually have poor prognosis
78 largely because of late diagnosis of the disease [2]. Despite advances in surgical techniques combined with
79 various treatment modalities, such as radiotherapy and chemotherapy, the overall 5-year survival rate remains at
80 20-30% [3]. To alleviate this clinical situation, the development of new treatment modalities, diagnostic
81 technologies and preventative measures is required, which cannot be accomplished without understanding the
82 underlying mechanisms of esophageal carcinogenesis. The development of gene microarray technology allows
83 comprehensive comparison of gene expression profiles in various pathophysiological processes, such as
84 enabling the comparison of gene profiles between cancer and normal conditions. In the present study, we took
85 advantage of this technology to identify differentially expressed genes in cancerous and non-cancerous
86 conditions in esophagus, followed by further validation for the involvement of these genes in ESCC.
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89 **Materials and Methods**

90 *HKESC-4 cell line and clinical specimens for microarray.* HKESC-4, a human ESCC cell line of Chinese origin,
91 was established previously in our laboratory and the culture conditions for this cell line were as described
92 elsewhere [4]. Cultured HKESC-4 cells were harvested at 80% confluency at passage 30 for extracting RNA.
93 Parental tumoral tissue (T), from which HKESC-4 cell line was derived, and its corresponding non-tumoral
94 epithelium (N) and lymph node containing metastatic carcinoma (LN) were isolated during esophagectomy and
95 snap-frozen until their use for RNA extraction. The extracted RNA of the cell line (HKESC-4) and clinical
96 specimens (T, N and LN) was subjected to gene microarray.
97

98 *Clinical tissues and ESCC cell lines for quantitative real time polymerase chain reaction (qPCR).* In addition to
99 the clinical specimens for gene microarray as mentioned above, three non-tumoral tissues were obtained from
100 esophageal epithelium at least 5 cm away from the tumor from other patients during surgical resection (Non-T).
101 Apart from HKESC-4 cell line, HKESC-1, HKESC-2, HKESC-3 and SLMT-1 cell lines were included and used
102 as described elsewhere [4-6]. These cells were cultured in minimal essential medium (Invitrogen, Carlsbad, CA,
103 USA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin. Cell cultures
104 were maintained in a humidified incubator at 37°C containing 5% carbon dioxide (CO_2). Monolayer cells at
105 80% confluency were harvested and used for RNA extraction.
106

107 *RNA extraction for qPCR.* RNA extraction was performed as described [7]. In brief, 1 ml TRIzol reagent
108 (Invitrogen) was used to lyse tissues or cells for RNA extraction. Chloroform was used for phase separation.
109 After centrifugation, the upper aqueous phase with RNA was collected and transferred to RNase-free tubes
110 containing 0.5 ml isopropyl alcohol for RNA precipitation. RNA pellets were then washed with 75% RNase-free
111 ethanol. Finally, RNA pellets were dissolved in RNase-free water. DNase I digestion was performed before the
112 concentration of RNA was determined by measuring its absorbance at 260 nm and A_{260}:A_{280} ratio
113

114 *Gene microarray.* RNA of HKESC-4 cells and clinical specimens were subjected to gene expression profiling
115 using GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). This GeneChip
116 enables the analysis of over 47,000 human transcripts and variants. The whole procedure of RNA quality
117 control, microarray labeling, GeneChip hybridization and data acquisition was performed at the Genome
118 Research Centre, The University of Hong Kong, Hong Kong, PRC under standardized condition. The statistical
119 analysis to identify differentially expressed genes was performed using MicroArray Suite software (Affymetrix).
120

121 *Synthesis of complementary DNA (cDNA).* RNA was reverse-transcribed into cDNA using High Capacity cDNA
122 Reverse Transcription Kit (Applied Biosystems, Invitrogen), according to the manufacturer's protocol. Briefly,
123 DNase-treated RNA was diluted with RNase-free water to reach a final concentration of 250 ng in 10 µl. Diluted
124 RNA was then mixed with 2 µl 10X RT buffer, 0.8 µl 25X dNTP Mix (100 mM), 2 µl 10X RT Random
125 Primers, 1 µl MultiScribe Reverse Transcriptase (50 U/µl), 1 µl RNase Inhibitor and 3.2 µl nuclease-free water.
126 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
127 inactivate the activities of the reverse transcriptase and to completely denature the template.
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qPCR. The procedure for qPCR was followed as described elsewhere [8, 9]. In brief, qPCR was performed using cDNA of each sample, gene-specific primers (Table 1) and Platinum SYBR Green qPCR SuperMix-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 test was 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 0.2 M citrate buffer (pH 6) in a microwave oven at 95°C for 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 1X TBS 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 biotinylated anti-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 expression of HMGA2 was examined under a light microscope.

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160 **Results**

161 *Identification of differentially expressed genes using gene microarray.* Gene microarray analysis revealed 3,081
162 genes, 4,027 genes and 3,590 genes having more than 2-fold induction in T, LN and HKESC-4 cells when
163 compared to N. For down-regulated genes with less than 2-fold difference, 4,808 genes, 6,052 genes and 5,846
164 genes were found in T, LN and HKESC-4 cells in comparison with N. For those up-regulated genes in T, LN
165 and HKESC-4, 43 of them had more than 10-fold induction (Table 2). Among them, 34 genes (*CAMK2A*,
166 *CART1*, *CCNA1*, *COCH*, *FLJ33516*, *FMN2*, *FOXD1, FOXG1B, GPR*, *HMGA2*, *HOXC10*, *HOXC11*, *HTR2C*,
167 *IMP-3*, *LOC163782*, *MAGEB2*, *MFAP2*, *MGC17986*, *MGC27005*, *NBEA*, *NKX2-2, PCDHB5*, *PEG10*, *PFN2*,
168 *POPDC3*, *PPFIA1*, *PRAME*, *SAGE1*, *SHANK2*, *SIX1*, *SLCO1B3*, *SYT1, TP53TG3* and *WISP3*) were first
169 identified to be overexpressed in ESCC, while 13 of them (*DKK1*, *EGFR, EMS1*, *GAL*, *HCG4*, *LAMC2*,
170 *MAGEA1, MAGEA4, MAGEA11*, *MMP13*, *PTHLH*, *ZIC* and *ZNF595*) have previously been reported to be
171 overexpressed in ESCC (Table 3).
172

173 *Gene expression of HMGA2.* High expression of *HMGA2* in N, T, LN and HKESC-4 observed in gene
174 microarray was confirmed using qPCR, such that the relative intensities of *HMGA2* expression in N, T, LN and
175 HKESC-4 obtained using these two methods were comparable (fold change by gene microarray: 1, 31, 63 and
176 18 *versus* 1, 26, 49 and 33 by qPCR). Moreover, increased gene expression of *HMGA2* was also detected in four
177 ESCC cell lines (HKESC-1, HKESC-2, HKESC-3 and SLMT-1). Significantly higher gene expression on
178 average of 20-fold of *HMGA2* was noted in ESCC cell lines when compared to N and Non-T (Figure 1A).
179

180 *Gene expression of PEG10.* An over-expression of *PEG10* in ESCC observed in gene microarray was confirmed
181 using qPCR. The relative intensities of *PEG10* in N, T, LN and HKESC-4 cells were 1, 20, 28 and 28 by gene
182 microarray, while their relative intensities were 1, 45, 148 and 269 by qPCR, respectively. However, no
183 significant difference in the average gene expression of *PEG10* was detected in tested ESCC cell lines when
184 compared to N and Non-T, despite their having an average of 100-fold induction in gene expression of *PEG10*
185 (Figure 1B).
186

187 *Gene expression of SHANK2*

188 High gene expression of *SHANK2* in ESCC observed in gene microarray was confirmed using qPCR. The
189 relative intensities of *SHANK2* in N, T, LN and HKESC-4 cells were 1, 30, 20 and 20 by gene microarray and 1,
190 21, 11 and 19 by qPCR, respectively. No significant difference in the gene expression of *SHANK2* was detected
191 in ESCC cells when compared to N (Figure 1C).
192

193 *Gene expression of WISP3.* High gene expression of *WISP3* in ESCC detected using gene microarray was
194 confirmed using qPCR. The relative intensities of gene expression of *WISP3* in N, T, LN and HKESC-4 cells
195 were 1, 37, 53 and 26 by gene microarray and 1, 152, 117 and 117 by qPCR, respectively. A significant increase
196 in the gene expression of *WISP3* was detected in HKESC-2 cells. An average of 100-fold higher gene
197 expression of *WISP3* was found when compared to N and Non-T (Figure 1D).
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Protein expression of HMGA2 in ESCC tissues. Immunohistochemical data showed 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.

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-tumoral esophageal epithelial tissues. The current findings showed that overexpression of *HMGA2* gene appears to be a consistent feature in ESCC. The protein expression of HMGA2 was further validated in ESCC specimens by immunohistochemistry. 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 of transcriptional 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 [13]. However, overexpression of HGMA2 has been reported in various types of human cancer including of the pituitary [14], oral cavity [15], lung [16], breast [17], pancreas [18], and nerves [19]. 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 [15]. 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 [20].

Overexpression of *HMGA2* gene leads to pituitary adenomas in mice. The mechanism has been described by Fedele *et al.* [21]. 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 [21]. 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 [22]. 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 [23, 24], resulting in the release of E2F1, the transcription of its target genes is activated [25]. This allows cells to progress toward S phase. The overexpressions of pRB [26] and E2F1 [27] 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.

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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-tumoral esophageal 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’
-Actin	5’-GCTCGTCGTCGACAACGGCTC-3’ 5’-CAAACATGATCTGGGTCATCTTCTC-3’

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280 Table 2. Genes with >10-fold higher expression in esophageal squamous cell carcinoma tissues and cells
281 studied
282

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

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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

283 * Relative to morphologically normal esophageal epithelium

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284
285 Table 3. Genes found to have overexpression in ESCC in previous studies.
286

Gene Symbol	Description	Reference
DKK1	Overexpression of *DKK1* gene in the distal squamous esophageal mucosa in patients with esophagitis	[28]
EGFR	Overexpression of *EGFR* in ESCC and its correlation with depth of tumor invasion	[29]
EMS1	Association of amplification and overexpression of *EMS1* with lymph node metastasis in ESCC	[30]
GAL	Distribution of *galanin* (GAL) immunoreactive nerve bundles and scattered nerve fibres in esophageal carcinoma	[31]
HCG	High expression of *HCG* expression in patients with lymph node metastasis and its correlation with infiltration and metastasis	[32]
LAMC2	Co-expression of *LN-5 gamma2* (*LAMC2*) and *EGFR* is closely related to the progression and poor prognosis of ESCC	[33]
PTHLH	High level of serum parathyroid hormone-related protein (*PTHLH*) in esophageal carcinoma	[34]
MAGE-A, MMP13, ZNF595	High expression of *MAGE-A*, *MMP13* and zinc finger proteins in ESCC	[35]

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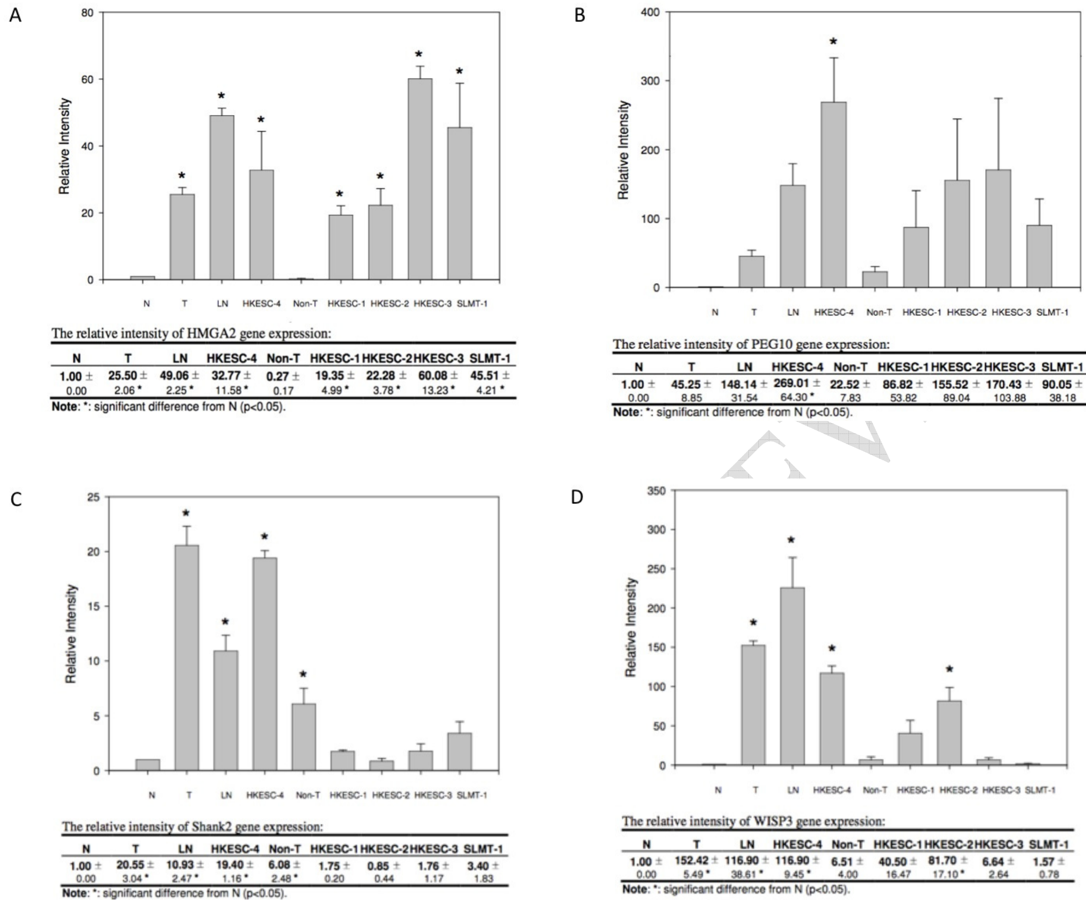
Figure 1. Gene expression of *HMGA2* (A), *PEG10* (B), *SHANK2* (C) and *WISP3* (D) relative to β-actin in various tissues and cell lines.



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).

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).

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).

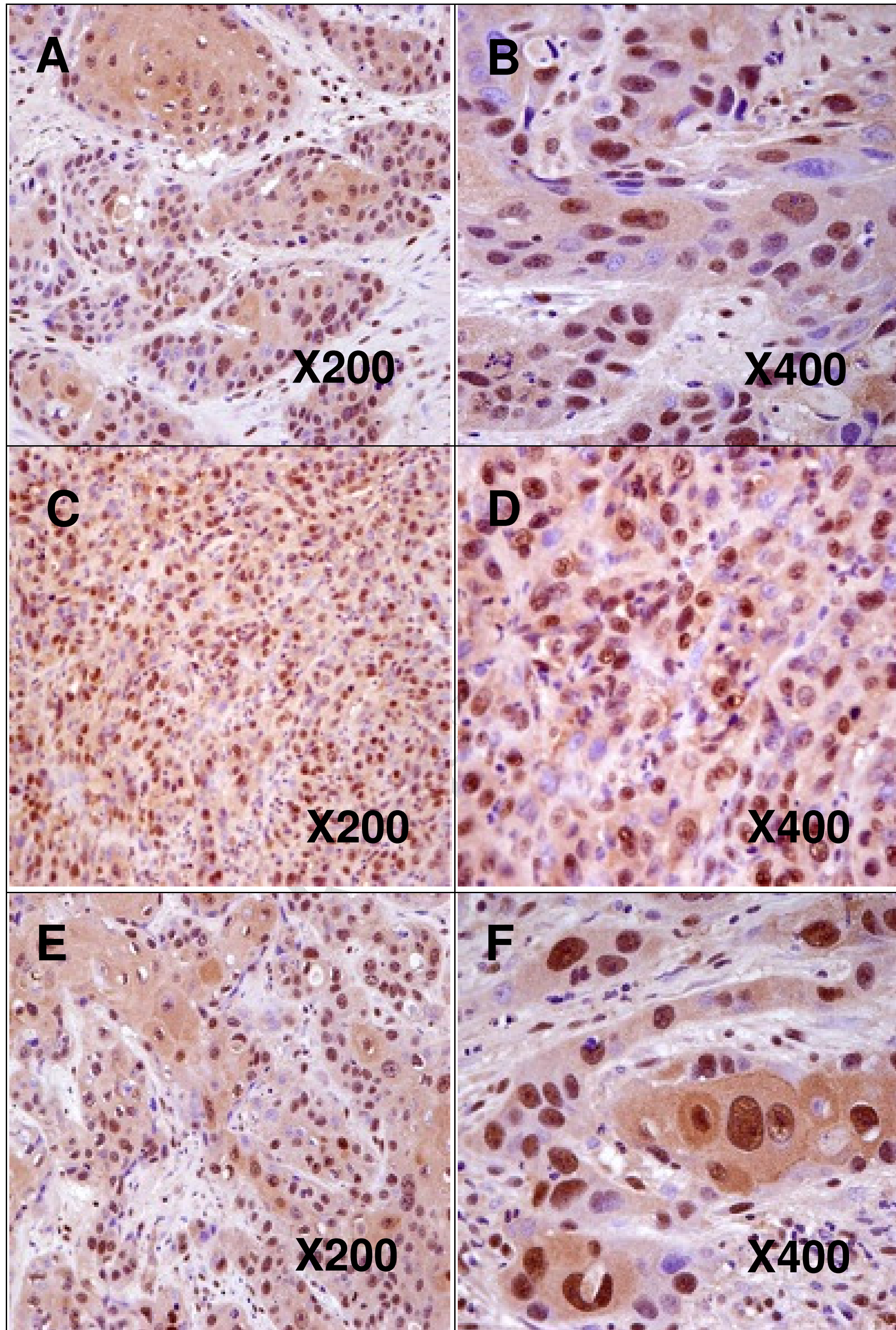
The relative intensity of WISP3 gene expression:

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.

302
303 Figure 2. Immunohistochemical staining of HMGA2 expression in ESCC tissues.
304



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