

# The involvement of H19 non-coding RNA in stress: Implications in cancer development and prognosis

## Research Article

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**Abbreviations:** active cyclin dependent kinase 2, (CDK2); angiopoietin 1 receptor precursor, (TIE-2); c-jun N-terminal kinase, (JNK); dimethyl sulphoxide, (DMSO); extracellular signal-regulated protein kinase, (ERK); fas-activated serine, (FAS); fetal calf serum, (FCS); fibroblast growth factor receptor 1 precursor, (FGFR1); Focal adhesion kinase, (FAK); Hanks' Balanced Salt Solution, (HBSS); lipid-activated protein kinase 2, (PRK2); mitogen-activated protein kinase and extracellular signal-regulated protein kinase, (MEK2); mitogen-activated protein, (MAP); NF-kB-inducing kinase, (NIK); nuclear factor -B, (NF- B); phytohemagglutinin M, (PHA); placenta growth factor, (PIGF); placental plasminogen activator inhibitor 2, (PAI-2); polymerase chain reaction, (PCR); Protein kinase C , (PKCA); protein kinase C- , (PKC- ); receptor-associated kinase, (IRAK IL1); reverse transcriptase-polymerase chain reaction, (RT-PCR); Tumor necrosis factor- , (TNF- ); Urokinase plasminogen activator receptor, (uPAR); vascular endothelial growth factor receptor 1, (VEGFR1); vascular permeability factor/vascular endothelial growth factor, (VPF/VEGF)

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## Summary

The H19 gene is an imprinted gene expressed from the maternal allele. It is known to function as an RNA molecule, cDNA microarray hybridization was used in an attempt to identify novel kinases participating in cellular response to hypoxia and serum deprivation. The expression of H19 RNA was examined in embryonic cells (Human amniocytes) that normally express H19 RNA basal level. At low serum (0.1% FCS) medium or hypoxia: 100 $\mu$ M CoCl<sub>2</sub>; or both: without serum (0.1% FCS) and 100 $\mu$ M CoCl<sub>2</sub> for 16hr the fold increase of H19 RNA expression was: 1.9  $\pm$  0.11, 1.73  $\pm$  0.2 and 2.0  $\pm$  0.18 folds respectively. Significant increase in expression and induced (up) expression of certain genes were observed in TA31 cell line that highly expresses H19 RNA. Using the human cDNA atlas microarray, we detected differentially expressed genes modulated by the presence of H19 RNA in certain conditions: serum deprivation, hypoxia and both serum deprivation and hypoxia which may resemble the stress conditions in cancer. Some of the key genes that had increased or induced (up) expression mainly in serum deprivation are: CDK2, FGFR1, IRAK, JNK1, uPAR and PRK2. In hypoxia the key genes are PKC- , cot-proto oncogene, PKC- , FAK and MEK2. In serum deprivation and hypoxia these genes are: Tie2, JNK2, ERK2 and VEGFR1. Using Atlas Array and observing the genes that had increased or induced (up) expression, a good indication for certain genes and pathways was found to be involved in tumor progression and angiogenesis. The major angiogenesis genes include FGFR1, VEGF, TIE2, uPA, and PKC- . Other signal molecules associated with the invasive and migratory potential include JNK2, uPAR and FAK.

## I. Introduction

H19 is the first imprinted gene with no protein product described to have oncofetal properties (Ariel et al, 1997). Little is known about the function of this imprinted gene, though it is expressed abundantly in the human placenta and in several embryonic tissues. A gene lying in

exons with a very low mutation rate and having significant expression levels in certain human cells and tissues, must have a function, if not having several vital functions (Hurst and Smith, 1999).

H19 expression increases in certain conditions and tissues (Tycko and Morison, 2002). It increases in the

carotid artery after injury, suggesting its role during wound healing. During embryogenesis H19 RNA level is highly elevated. Previous studies showed that H19 fulfills an important role in the process of tumorigenesis (Looijenga and Verkerk, 1997).

H19 is expressed abundantly in many cancer types, but is only marginally expressed in nearly all normal adult tissues. In some cases of breast adenocarcinoma with poor prognosis, H19 is over expressed in epithelial cells (Lottin et al, 2002). Our observations that ectopic expression of H19 RNA alters expression profiles of (certain) genes involved in metastasis and blood vessel development, support the notion of a role for this gene in tumor invasion and angiogenesis. This role seems to be triggered by stress conditions that accompany tumor growth (better to be in Discussion not here. It is especially noteworthy that many of the genes modulated by H19 RNA are also hypoxia responsive (Ayesh et al, 2002) The realization that a lot of us carry in situ tumors (microscopic tumors), but do not develop the disease, suggests that these microscopic tumors are mostly dormant and need additional signals to grow and become lethal tumors (Folkman and Kalluri, 2004). H19 is considered a tumor marker that combines prognostic and predictive value in patients with refractory superficial cancer (Ariel et al, 2000). The search for key genes which convert the non-lethal tumors into the expanding mass of tumor cells that is potentially lethal to an individual became a very important issue.

To investigate more about the function of H19, we transfected cells from the bladder carcinoma cell line T24P, which does not express H19, with an episomal construct in which H19 expression is under the control of the cytomegalovirus promoter in either a sense full-length cDNA construct (TA31 cells), or an anti-sense construct covering 800 bp that extended from the 3' end direction (TA11 cells). We aimed to identify kinases and genes that showed altered expression between the TA31 (H19+) and TA11 (H19-) cell lines with the Atlas human cDNA expression array, containing cDNA from 350 all kinases. We also compared the effect of the presence of H19 RNA on the proliferation capacity of cells, and plotted out key genes that were noticeably up regulated or over expressed both in normal and poor serum conditions.

Some of the differentially expressed kinases are among those promoting invasion, migration, angiogenesis and notably apoptosis. These findings and results support the suggestion of H19 functioning in cancer progression by overcoming stress conditions thereby enabling cells to survive and proliferate.

## II. Materials and methods

### A. Cell culture

The human bladder carcinoma cell line T24P was obtained from the American Type Culture Collection (Manassas, VA). Cells from the T24P cell line were stably transfected with an episomal vector that has an H19 full-length cDNA placed in either the sense direction, creating TA31, or the antisense direction (800 bp from 3' end), creating TA11. The cells were grown as previously described (Kopf et al, 1998). For serum deprivation and hypoxia, these cells were grown in low serum conditions (0.1% fetal calf serum (FCS)) medium or hypoxia: 100µM CoCl<sub>2</sub> (Wang et al, 2000); or both: in low serum

conditions (0.1% FCS) and 100µM CoCl<sub>2</sub> for 16hr before RNA extraction.

### 1. Human amniocytes

Human amniocytes were cultured in sterile flasks grown to confluence in RPMI medium supplement. It contained 10% FCS, 1% Penicillin/ Streptomycin, 1% L-glutamate, and 1.3% phytohemagglutinin M(PHA). They were grown at 37°C in a humidified incubator (95% air, 5% CO<sub>2</sub>), according to cytogenetics laboratory procedure manual (Genetics Division LAC/USA medical center,1990). When the cells reached confluence, they were washed with Hanks' Balanced Salt Solution (HBSS). Then trypsinized with EDTA-trypsin, and neutralized with Bio-amf media (biological industries, Israel). The media with the cultured cells were collected and sub-cultured in 4 different flasks according to the previous culture conditions for 48h. Later on, the flasks were washed with HBSS and incubated for 16h with four different types of media. These media are: medium A: the same medium mentioned above; medium B: low serum conditions (0.1% FCS); medium C: 100µM CoCl<sub>2</sub>; medium D: low serum conditions (0.1% FCS) and 100µM CoCl<sub>2</sub>.

### B. RNA Extraction and RT-PCR

Conditional media were collected; the cells were lysed, and neutralized. Then total RNA was extracted by RNA STAT-60 (TEL-TEST INC, Friends wood, TX) according to manufacturer instructions. For RT-PCR reaction, the synthesis of cDNA was performed using p(dT)<sub>15</sub> primer (Boehringer, Mannheim, Germany) to initiate reverse transcription of 2 µg total RNA with 400U of M-MLV reverse transcriptase (GibcoBRL® Gaithersburg, MD).

The cDNA was used as a template for PCR to amplify the tested genes, H19 and Histone H3.3. The amplification was performed in a final volume of 25 µl reaction mixture. It contained 2µl of cDNA, 0.625 units of *Taq* DNA polymerase (Takara, Otsu, Japan), its 1X buffer (50 mM KCl, 2 mM MgCl<sub>2</sub>, 10 mM Tris-HCl), 0.2 mM dNTP mix, and 0.15µg of each primer. DMSO (4.5%) was also used in the amplification of H19 transcript. Thermal cycling parameters for H19 were: denaturation at 98 °C for 15sec, annealing at 58°C for 30sec, and extension at 72°C. In all the PCR assays, the number of cycles was calibrated to ensure that PCR amplification was in the linear phase. Each PCR was repeated 3 times. The integrity of the cDNA was assayed by PCR analysis with the ubiquitous cell cycle independent histone variant H3.3, as described by Futscher et al, (1993). Photographs of the PCR products were scanned with a PowerLook II scanner and quantified with ImageGauge version 3.41 software (Fuji Photo Film Co., Tokyo, Japan).

### C. The custom Atlas array

The custom Atlas kinase array (Clontech Labs Inc) includes 359 human complementary DNAs of known kinases and phosphatase genes, divided into categories. In addition, the array includes 9 housekeeping genes for internal control of gene expression; genomic DNA spots as orientation markers and controls of labeling efficiency; and negative controls immobilized in duplicate dots on a nylon membrane.

### D. RNA labeling and hybridization

The Atlas array kit contains all necessary ingredients for RNA labeling, probe purification, and hybridization. Total DNA-free RNA (5 µg) from each tissue sample were labeled by <sup>32</sup>-P-dATP. The complementary DNA probe was purified on a special column provided in the kit. Equal amounts of labeled probe (about 10<sup>7</sup> cpm) for each cell line were hybridized to the array.

After several washings the arrays were exposed to radiographs at  $-80^{\circ}\text{C}$  for 7, 10, and 16 hours.

The whole analysis was carried out twice. The difference between pattern and degree of gene expression was calibrated using household genes in the two independent experiments.

### E. RNA identification and comparison

Signals of exposure were scanned and quantified with software for digital image analysis (Atlas-image, v. 2; Clontech Labs Inc). This program is designed to compare gene expression profiles and generate a detailed report. Briefly, after alignment of the 2 arrays to the grid template, the background calculation was performed. The program generates intensity values (the average of the total signal from the left and right spots in double-spotted arrays) and the normalization coefficient is calculated first for array 1 and then applied to the adjusted intensity of each of the genes on array 2. The adjusted intensity for a gene is the intensity value minus background value multiplied by the normalization coefficient. The ratio and difference values were calculated.

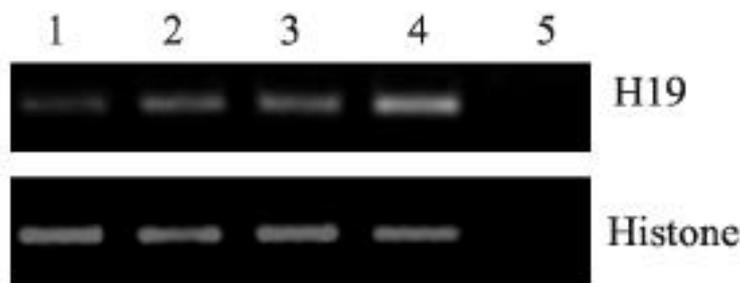
Comprehensive information on the genes included in the array is found at Clontech Labs Inc's Atlas info bioinformatics database (atlasinfo.clontech.com).

## III. Results

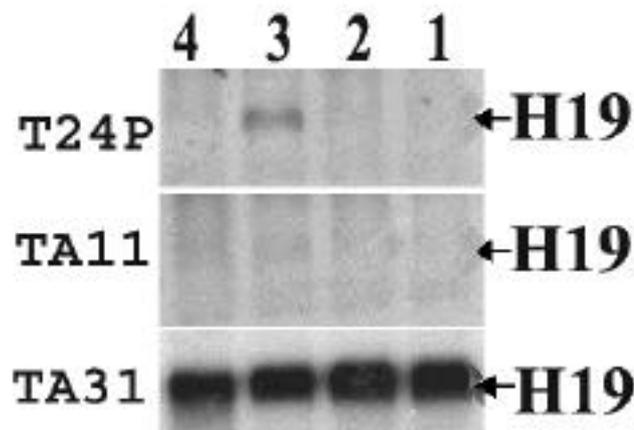
### A. H19 expression at different stress conditions

H19 expression in human amniocytes: the level of H19 RNA was examined in embryonic cells (Human amniocytes) that normally express H19 at basal level. The change in H19 RNA expression was measured by RT-PCR after different stress conditions and the results are as shown below. **Figure 1** shows that there is an increase in H19 RNA level in low serum (0.1% FCS) medium or hypoxia:  $100\mu\text{M}$   $\text{CoCl}_2$ ; or both: low serum conditions (0.1% FCS) and  $100\mu\text{M}$   $\text{CoCl}_2$  for 16h. The increase of H19 RNA expression was:  $1.9 \pm 0.11$ ,  $1.73 \pm 0.2$  and  $2.0 \pm 0.18$  folds respectively.

H19 expression in T24P, TA 11 and TA 31 cell lines was examined at low serum (0.1% FCS) medium, or hypoxia:  $100\mu\text{M}$   $\text{CoCl}_2$ ; or both: low serum conditions (0.1% FCS) and  $100\mu\text{M}$   $\text{CoCl}_2$  for 16h by northern blot. As shown in **Figure 2**, the H19 level was slightly increased in T24p cell line at hypoxia ( $100\mu\text{M}$   $\text{CoCl}_2$ ), while no H19 induction in TA 11 cell line, which contains the plasmid that expresses the anti-sense for H19.



**Figure 1.** H19 expression in human amniocytes. H19 RNA expression in usual medium (10% FCS) lane 1, low serum (0.1% FCS) medium lane 2, hypoxia:  $100\mu\text{M}$   $\text{CoCl}_2$  lane 3; or both: low serum conditions (0.1% FCS) and  $100\mu\text{M}$   $\text{CoCl}_2$  lane 4 for 16h and blank lane 5. The increase of H19 RNA expression was:  $1.9 \pm 0.11$ ,  $1.73 \pm 0.2$  and  $2.0 \pm 0.18$  folds respectively.



**Figure 2.** Northern blot analysis of H19 expression in T24P, TA11 and TA31 cell lines at normal and different stress conditions. The H19 RNA expression in normal conditions lane 1, in low serum (0.1% FCS) medium lane 2, or hypoxia:  $100\mu\text{M}$   $\text{CoCl}_2$  lane 3; or both: low serum conditions (0.1% FCS) and  $100\mu\text{M}$   $\text{CoCl}_2$  for 16hr lane 4, examined by northern blot in the three cell line: T24p cell line, TA 11 cell line, TA 31 cell line.

### 1. Gene expression analysis

The results of microarray gene analysis after different stress conditions were as follows: The genes listed in these **Tables (1, 2, 3)** are those increased significantly (more than 1.5 fold) or induced (up) in TA31 cell line compared to TA 11 and T24p cell lines. **Table 1** contains the genes with increased and induced (up)

expression at low serum (0.1% FCS) medium. **Table 2** contains the genes that increased or induced expression (up) in hypoxia (100µM CoCl<sub>2</sub>); **3** contains the genes that increased or induced (up) at double stress conditions (low serum conditions (0.1% FCS) and 100µM CoCl<sub>2</sub> for 16h.

**Table 1.** Genes that had increased or induced (up) expression with a ratio of more than 1.5 fold at low serum (0.1% FCS) medium in TA 31 cell line compared to TA11 and T24p cell lines

B6e	2.31673	serine/threonine-protein kinase PCTAIRE 3 (PCTK3)	X66362
B5a	2.346823	Ribose phosphophate pyrophosphokinase	M57423
A7a	2.531401	fibroblast growth factor receptor1 precursor (FGFR1)	X66945
C2d	2.64694	checkpoint kinase 1 (CHK1)	AF016582
C1a	2.702954	hint protein; protein kinase C inhibitor 1 (PKCI1)	U51004
B1a	3.088425	Diacylglycerol kinase	AF064771
B3k	3.276213	protein kinase A anchoring protein	AF037439
D3e	3.287641	CDC28 protein kinase 2	AA010065
A2m	3.387337	DRAK2	AB011421
A6e	3.873097	neurotrophic tyrosine kinase receptor type 1 (NTRK1)	X03541
A1f	5.618388	cyclin-dependent protein kinase 2 (CDK2)	M68520
A7d	5.633224	protein kinase C polypeptide (PKC- )	M22199
A3f	Up	c-jun N-terminal kinase 1 (JNK1)	L26318
A3n	Up	Protein-tyrosine kinase transmembrane	M97639
A4d	Up	cyclin-dependent kinase 10 (CDK10)	L33264
A4k	Up	mitogen-activated protein kinase kinase 6 (MAP kinase kinase 6)	U39657
A5d	Up	urokinase-type plasminogen activator precursor (uPAR)	M15476
A5m	Up	SHK1 kinase binding protein 1	AF015913
A6a	Up	angiopoietin 1 receptor precursor	L06139
A7n	Up	Muscle specific tyrosine kinase receptor	AF006464
B1b	Up	Selenide water dikinase 1	U34044
B1n	Up	Lipid-activated protein kinase 2 (PRK2)	U33052
B3d	Up	cell division protein kinase 4	M14505
B4m	Up	ribosomal protein S6 kinase II 3 (S6KII- 3)	U08316
B5l	Up	cAMP-dependent protein kinase -catalytic subunit (PKA C- )	M34182
B5m	Up	serine/threonine-protein kinase (NEK2)	U11050
B6h	Up	Bruton's tyrosine kinase (BTK)	U10087
B7a	Up	A kinase anchor protein	U17195
C2c	Up	serine/threonine-protein kinase (NEK3)	Z29067
C3f	Up	adenylate kinase 3 (AK3)	X60673
C5c	Up	phosphatidylinositol 3-kinase catalytic subunit delta isoform	U86453
C5e	Up	protein tyrosine kinase	U02680
C5j	Up	activin receptor type I precursor (ACTRI)	L02911
C7b	Up	serine/threonine protein kinase (SAK)	Y13115
D6f	Up	serine/threonine-specific protein kinase minibrain	U58496

**Table 2.** Genes that that had increased or induced (up) expression with a ratio of more than 1.5 fold at hypoxia in TA 31 cell line compared to TA11 and T24p cell lines

Gene code	Ratio	Protein/gene	Gene bank accession
B1i	1.505267	ephrin type-B receptor 1 precursor	L40636
A7d	1.510266	Protein kinase C polypeptide (PKC- )	M22199
A1m	1.512111	ntak protein (neural and thymus derived activator for erbb kinases	AB005060
B6k	1.52491	ribosomal protein S6 kinase II 1 (S6KII- 1)	L07597
A6d	1.528496	Placental plasminogen activator inhibitor 2 (PAI-2)	M18082
D3h	1.544107	mitogen-activated protein kinase 9	L31951
B6e	1.555473	serine/threonine-protein kinase PCTAIRE 3 (PCTK3)	X66362

C2d	1.568302	checkpoint kinase 1 (CHK1)	AF016582
D1d	1.596408	protein kinase C (PKC- )	Z15108
C3k	1.600221	6-phosphofructokinase	D25328
A4c	1.604381	focal adhesion kinase (FAK)	L13616
B7f	1.623765	NIK serine/threonine protein kinase	Y10256
A7c	1.635135	protein serine/threonine kinase (STK1)	L20320
A2m	1.641395	DRAK2	AB011421
B2j	1.671949	nucleoside diphosphate kinase A (NDKA)	X17620
D4a	1.689245	calmodulin (CALM)	J04046
B2e	1.805216	cell division control protein 2 homolog (CDC2)	X05360
C5k	1.833114	putative diacylglycerol kinase eta (DAG kinase eta)	D73409
B3l	1.882828	cAMP-dependent protein kinase I regulatory subunit (PRKAR1)	M33336
D3e	1.933686	CDC28 protein kinase 2	AA010065
C4l	1.940017	guanine nucleotide-binding protein subunit 2-like protein 1 (GNB2L1)	M24194
B3k	2.143857	protein kinase A anchoring protein	AF037439
B1c	2.209804	tyrosine-protein kinase ctk	L18974
A6e	2.553698	neurotrophic tyrosine kinase receptor type 1 (NTRK1)	X03541
D1b	2.59454	Creatin kinase B chain	L47647
B2k	2.730018	STE20-like kinase 3 (MST3)	AF024636
B4k	2.73525	cot proto-oncogene	D14497
C4i	2.925536	mevalonate kinase	M88468
C7d	3.366751	serine/threonine protein kinase minibrain homolog (DYRK)	D86550
A6a	Up	angiopoietin 1 receptor precursor (TIE-2)	L06139
B1g	Up	mitogen-activated protein kinase kinase 2 (MAP kinase kinase 2)	L11285
B3d	Up	cell division protein kinase 4; cyclin-dependent kinase 4 (CDK4)	M14505
B5c	Up	tyrosine-protein kinase itk/tsk	D13720
B5l	Up	cAMP-dependent protein kinase gamma-catalytic subunit	M34182
B5m	Up	serine/threonine-protein kinase (NEK2)	U11050
B6i	Up	serine/threonine-protein kinase PLK1 (STPK13)	U01038
B6l	Up	c-ros-1 tyrosine-protein kinase proto-oncogene	M34353
B6m	Up	STE20-like kinase (MST2)	U26424
B7a	Up	A-kinase anchor protein	U17195
C2n	Up	mitochondrial thymidine kinase 2	U77088
C3f	Up	adenylate kinase 3 (AK3)	X60673
C3i	Up	phosphomevalonate kinase (PMKase)	L77213
C5h	Up	dual-specificity protein phosphatase 9	Y08302
C5j	Up	activin receptor type I precursor (ACTRI)	L02911
C5n	Up	1D-myo-inositol-trisphosphate 3-kinase B	X57206
C6e	Up	MAP kinase-activating death domain protein	U77352
C6h	Up	myotonic dystrophy protein kinase-like protein	Y12337
C7e	Up	serine kinase 9 (SRPK2)	U88666
D1c	Up	calcium/calmodulin-dependent protein kinase type II	U50359

**Table 3.** Genes that had increased or induced (up) expression with a ratio of more than 1.5 fold at low serum (0.1% FCS) medium and hypoxia in TA 31 cell line compared to TA11 and T24p cell lines

Gene code	Ratio	Protein/gene	
C4l	1.576539	guanine nucleotide-binding protein subunit 2-like protein 1 (GNB2L1)	M24194
C6j	1.590471	myotonin-protein kinase; myotonic dystrophy protein kinase (MDPK)	L19268
A1h	1.680315	DNA-dependent protein kinase (DNA-PK)	U35835
B7c	1.715803	cell division protein kinase 8 (CDK8)	X85753
D4a	1.73196	calmodulin (CALM)	J04046
A7a	1.762276	fibroblast growth factor receptor1 precursor (FGFR1)	X66945
B4k	1.7795	cot proto-oncogene	D14497

C5k	1.864683	putative diacylglycerol kinase eta (DAG kinase eta)	D73409
A2m	2.046678	DRAK2	AB011421
B5h	2.072661	ephrin type-A receptor 5 precursor (EHK1)	X95425
D3h	2.303345	mitogen-activated protein kinase 9	L31951
A1j	2.307026	vascular endothelial growth factor receptor 3 precursor (VEGFR3); flt-4	X68203
C5i	2.396003	phosphatidylinositol 3 kinase catalytic subunit isoform	X83368
A1m	2.504037	ntak protein (neural and thymus derived activator for erbb kinases)	AB005060
D3e	2.706103	CDC28 protein kinase 2	AA010065
B2e	2.782374	cell division control protein 2 homolog (CDC2)	X05360
A7d	2.892207	protein kinase C polypeptide (PKC- )	M22199
A1f	2.971199	cyclin-dependent protein kinase 2 (CDK2)	M68520
C4i	4.024127	mevalonate kinase	M88468
A1d	Up	serine/threonine-protein kinase (STK2)	L20321
A2b	Up	related to receptor tyrosine kinase (RYK)	S59184
A2f	Up	protein kinase C (PKC- )	L07032
A2i	Up	fas-activated serine/threonine kinase (FAST)	X86779
A3b	Up	vascular endothelial growth factor receptor 1 (VEGFR1); Flt-1	X51602
B3d	Up	cell division protein kinase 4; cyclin-dependent kinase 4 (CDK4)	M14505
B5c	Up	tyrosine-protein kinase itk/tsk	D13720
B5l	Up	cAMP-dependent protein kinase -catalytic subunit (PKA C- )	M34182
B5m	Up	serine/threonine-protein kinase NEK2	U11050
B6b	Up	B-lymphocyte kinase (BLK)	Z33998
B7l	Up	deoxycytidine kinase	M60527
B7m	Up	58-kDa inhibitor of the RNA-activated protein kinase	U28424
C2c	Up	serine/threonine-protein kinase NEK3	Z29067
C4f	Up	phosphorylase B kinase catalytic subunit skeletal muscle isoform	X80590
D1h	Up	c-jun N-terminal kinase 1 (JNK1)	L26318
D2e	Up	hematopoietic progenitor kinase (HPK1)	U66464
D2f	Up	Adenylate kinase isoenzyme 2	U39945

## IV. Discussion

H19 was described to have oncofetal properties; it is expressed abundantly in the human placenta and in several embryonic tissues (Ariel et al, 1997). We cultured human amniocytes, which express H19 under normal conditions, at different stress condition i.e. hypoxia and serum stress (**Figure 1**). The increase in H19 expression (about 2 folds) in both serum deprivation and hypoxia was a strong indication that H19 is involved in the physiological response to different stress conditions.

The H19 level was slightly increased in T24p cell line at hypoxia (100µM CoCl<sub>2</sub>) as shown in **Figure 2**. While no H19 induction was found in TA 11 cell line, which contains the plasmid that expresses the anti-sense for H19, was found. It seems very likely that H19 RNA is involved in the induction of the expression of the kinases which increased significantly (more than 1.5 fold) or induced (up) in TA31 cell line compared to TA 11 and T24p cell lines.

Significant increase in expression and induced (up) expression of certain genes was observed in TA31 cell line which is H19+ and after growing these cells with stress conditions: which are serum deprivation, hypoxia and both serum deprivation and hypoxia together.

While taking a closer look at all the genes that had an increase or induced (up) expression in the hypoxia and serum stress conditions, which may resemble the stress conditions in cancer, certain important genes may be playing important roles in cell survival and the mitogenic activities of the tumor.

### A. Serum deprivation

Elevated expression of active cyclin dependent kinase 2 (CDK2) is critical for promoting cell cycle progression and unrestrained proliferation of tumor cells. CDK2 is retained in the cytoplasm of cells by serum deprivation (Bresnahan et al, 1997).

Apoptosis of human endothelial cells after growth factor deprivation and stress accompanied by cancer is associated with rapid and dramatic induced (up) expression of CDK2 activity. CDK2 activation, through caspase-mediated cleavage of cdk inhibitors, may be instrumental in the execution of apoptosis following caspase activation (Levkau et al, 1998). One of the stress kinases which we found to have induced (up) expression in serum deprivation is fibroblast growth factor receptor 1 precursor (FGFR1). FGFR1 may be a specific target for MMP2 on the cell surface, yielding a soluble FGF receptor that may modulate the mitogenic and angiogenic activities

of FGF. MMP2 is a key gene in angiogenesis (Levi et al, 1996).

Binding of interleukin-1 (IL1) to its receptor and by the association of IRAK (IL1 receptor-associated kinase), triggers activation of nuclear factor  $\kappa$ -B (NF- $\kappa$ B), a family of related transcription factors that regulates the expression of genes bearing cognate DNA binding sites such as PCNA which we also found to have induced (up) expression in pervious study (Ayesh et al, 2002). Another gene that had induced (up) expression was JNK1 (c-jun N-terminal kinase 1) which is involved in the initiation of the apoptosis process (Ch et al, 1996; Yu et al, 1996). JNK1 is activated by various stimuli, including UV light, Ha-Ras, TNF- $\alpha$  (Tumor necrosis factor- $\alpha$ ), IL-1 and CD28 costimulation (Derijard et al, 1994; Ch et al 1996). JNK1 phosphorylates Elk-1 on the same major sites recognized by ERK1/2 (extracellular-regulated kinase), thus potentiating its transcriptional activity (Cavigelli et al, 1995).

A critical gene involved in the mitogenic and invasive pathways and up regulated under stress conditions is uPA (Urokinase plasminogen activator). uPA is secreted as an enzymatically inactive proenzyme (pro-uPA). Urokinase plasminogen activator receptor (uPAR) mediates the binding of the zymogen, pro-uPA, to the plasma membrane where trace amounts of plasmin will initiate a series of events referred to as reciprocal zymogen activation where plasmin converts pro-uPA to the active enzyme, uPA, which in turn converts plasma membrane-associated plasminogen to plasmin (Dear et al, 1998, Plesner et al, 1997). Urokinase-type plasminogen activator receptor (uPAR) is known to play important roles in tumor cell migration, invasion, and metastasis (Ayesh et al, 2002). High levels of u-PA, PAI-1 (placental plasminogen activator inhibitor 2) and u-PAR in many tumor types predict poor patient prognosis (Fazioli and Blasi, 1994; Andreassen et al, 1997). PRK2 (lipid-activated protein kinase 2) is necessary for apoptosis, during FAS-induced apoptosis (Cryns et al, 1997) which can form a complex with adaptor proteins made up of src domains (Braverman and Quilliam, 1999).

## B. Hypoxia stress

Many key genes in the main pathway of tumorigenesis were found to have increased or induced (up) expression. The proliferation of new tumor cells instead could take place. PKC- $\delta$  (protein kinase C- $\delta$ ) is important in NF- $\kappa$ B activation (Folgueira et al, 1996) and takes a central position in TNF signal pathways acting as a molecular switch between mitogenic and growth inhibitory signals of TNF- $\alpha$ . (Muller et al, 1995). The role of TNF- $\alpha$  in angiogenesis is thought to be indirect through its ability to induce angiogenic factors. TNF- $\alpha$  mediates its action through NF- $\kappa$ B transcription factor (Ayesh et al, 2002). In serum-free media, NF- $\kappa$ B is activated promoting survival of cells while inhibiting PKC- $\delta$  results in cell death (Wang et al, 1999). PKC- $\delta$  was implicated in tumor angiogenesis (Pal et al, 1998). It is highly over expressed in tumors and is involved in apoptosis, angiogenesis, and several signal transduction pathways regulating

differentiation, proliferation or apoptosis of mammalian cells. Sp1 promotes the transcription of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), a potent angiogenic factor, by interacting directly and specifically with protein kinase C (PKC  $\delta$ ) isoform in renal cell carcinoma. PKC  $\delta$  binds and phosphorylates the zinc finger region of Sp1 (Pal et al, 1998).

One of the genes that had increased expression was cot-proto oncogene (c-cot/TPL-2) which encodes a MAP3K related serine threonine kinase and plays a critical role in TNF- $\alpha$  production. An increase in cot kinase expression promotes TNF- $\alpha$  promoter-driven transcription. Cot kinase is partially mediated by MEK/ERK kinase pathway which includes many up regulated genes in the stress conditions in order to survive. Cot kinase increases at least the AP-1 and AP-2 response elements (Ballester et al, 1998). It also plays a role in IL-2 production which is an important angiogenesis-associated secreted protein (Ballester et al, 1997). TPL-2 is a component of a signaling pathway that controls proteolysis of NF- $\kappa$ B1 p105 generating, at the end, active nuclear NF- $\kappa$ B. Furthermore, kinase-inactive TPL-2 blocks the degradation of p105 induced by (TNF- $\alpha$ ) (Belich et al, 1999). Cot assembles physically with NF- $\kappa$ B-inducing kinase (NIK) and phosphorylate it in vivo (Lin et al, 1999).

Protein kinase C- $\delta$  is the major protein kinase C isoenzyme of a signal transduction cascade regulating IL-2 receptor expression and which is over expressed in the experiment (Szamel et al, 1997). Focal adhesion kinase (FAK) is centrally implicated in the regulation of cell motility and adhesion (Zachary, 1997) and is induced by adhesion of cell surface integrins to extracellular matrix and other factors (Guan 1997; Zachary 1997). Activated FAK leads to its binding to a number of intracellular signaling molecules including Src, Grb2 and PI 3-kinase. Integrin signaling through FAK causes increased cell migration and potentially regulates cell proliferation and survival (Guan 1997). FAK is involved in the progression of cancer to invasion and metastasis and overexpression of FAK in tumor cells leads to a high propensity toward invasion and metastasis and increased cell survival under anchorage-independent conditions (Kornberg 1998). Other genes as MEK2 (MAPK and ERK kinase) contribute to the activation of the oxidative burst and phagocytosis, and participate in cytokine regulation of apoptosis in cells under stress (Downey et al, 1998).

## C. Serum deprivation and hypoxia stresses

Tie2 had an increased expression in all stresses and is known to play a role in tumor angiogenesis (Lin et al, 1998). Tie2 and its ligand angiopoietin-1 represent key signal transduction systems involved in the regulation of embryonic vascular development. The expression of these molecules correlates with phases of blood vessel formation needed in angiogenesis (Breier et al, 1997).

Three distinct groups of MAP kinases have been identified in mammalian cells (ERK, JNK, and p38).

These MAP kinases are mediators of signal transduction from the cell surface to the nucleus (Whitmarsh and Davis, 1996). Jun kinase (JNK1 and JNK2) is selectively mediating signal transduction of the pro-inflammatory cytokines IL-1 and TNF as well as of cellular stress (Uciechowski et al, 1996). JNK2 was found to be over expressed in both serum deprivations and hypoxia. IL-1, TNF, UV light and osmotic stress, are able to stimulate jun kinase activity (including JNK2) in humans (Uciechowski et al, 1996). JNK2 (also called Elk-1 activation domain kinase) phosphorylates the NH2-terminal activation domain of the transcription factor c-Jun, and the activity of JNK2 was approximately 10-fold greater than that of JNK1 (Sluss et al, 1994). JNK2 phosphorylates Elk-1 in extracts of UV-irradiated cells on the same major sites recognized by ERK1/2 that potentiate its transcriptional activity (Cavigelli et al, 1995).

The mitogen-activated protein (MAP) kinase also known as (ERK2) is proline-directed serine/threonine kinases that are activated in response to a variety of extracellular signals, including growth factors, hormones and, neurotransmitters. MAPK/ERK is a key molecule in intracellular signal transducing pathways that transport extracellular stimuli from cell surface to nuclei. MAPK/ERK has been revealed to be involved in the physiological proliferation of mammalian cells and also to potentiate them to transform and thus increase in amounts in tumor cells (Davis 1995). ERK2 is activated by many oncogenes, such as RAS and RAF, and they induce cell proliferation (Mishima et al, 1998).

Vascular endothelial growth factor receptor 1 (VEGFR1) also called FLT-1 gene encodes a transmembrane tyrosine kinase that is involved in angiogenesis and migration which is a high-affinity receptor for VEGF and placenta growth factor (PlGF). Flt-1 plays important roles in the angiogenesis required for embryogenesis and in monocyte/macrophage migration (Gerber et al, 1997). VEGF/PlGF functions via flt-1 in an autocrine manner to perform a role in invasion and differentiation (Shore et al, 1997). The Flt-1 receptor gene had direct induced (up) expression by hypoxia via hypoxia-inducible enhancer on the Flt-1 promoter (Gerber et al, 1997), and has been implicated in the regulation of blood vessel growth during angiogenesis (Breier et al, 1997; Cheung 1997). The VEGF signal transduction system has been implicated in the regulation of pathological blood vessel growth during certain angiogenesis-dependent diseases that are often associated with tissue ischemia, such as tumorigenesis (Shibuya et al, 1994; Breier 1997).

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