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• ESOPHAGEAL CANCER •

Differential gene expression between squamous cell carcinoma of esophageus and its normal epithelium; altered pattern of mal, akr1c2, and rab11a expression

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Abstract

AIM: To identify the altered gene expression patterns in squamous cell carcinoma of esophagus (ESCC) in relation to adjacent normal esophageal epithelium.

METHODS: Total RNA was extracted using SV total RNA isolation kit from snap frozen tissues of ESCC samples and normal esophageal epithelium far from the tumor. Radio-labeled cDNA were synthesized from equal quantities of total RNAs of tumor and normal tissues using combinations of 24 arbitrary 13-mer primers and three different anchoring oligo-dT primers and separated on sequencing gels. cDNA with considerable different amounts of signals in tumor and normal tissue were reamplified and cloned. Using southern blot, the clones of each band were controlled for false positive results caused by probable heterogeneity of cDNA population with the same size. Clones that confirmed differential expression by slot blot selected for sequencing and northern analysis. Corresponding full-length gene sequences was predicted using human genome project data, related transcripts were translated and used for various protein/motif searches to speculate their probable functions.

RESULTS: The 97 genes showed different levels of cDNA in tumor and normal tissues of esophagus. The expression of mal gene was remarkably down regulated in all 10 surveyed tumor tissues. Akr1c2, a member of the aldo-keto reductase 1C family, which is involved in metabolism of sex hormones and xenobiotics, was up-regulated in 8 out of 10 inspected ESCC samples. Rab11a, RPL7, and RPL28 showed moderate levels of differential expression. Many other cDNAs remained to further studies.

CONCLUSION: The mal gene which is switched-off in all ESCC samples can be considered as a tumor suppressor gene that more studies in its regulation may lead to valuable explanations in ESCC development. Akr1c2 which is up-regulated in ESCC probably plays an important role in tumor development of esophagus and may be proposed as a potential molecular target in ESCC treatments. Differential display technique in spite of many disadvantages is still a valuable technique in gene function exploration studies to find new candidates for improved ones like gene chips.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a predominant kind of cancer in the developing world as well as the north and northeast of Iran, which show age-standardized incidence rate of about 150 per 100 000 person-years for both sexes[1]. The exposure of esophageal cells to both exogenous agents such as food, alcohol, smoke and endogenous causes such as genetic and inflammation of esophagus tissue as well as the race and cultural habits have been accounted for high incidence in certain geographical regions[2]. Many genes including several oncogenes and tumor suppressor genes are deregulated in esophageal cancrcinoma[3, 4]. Some new publications reported gene expression profiles of normal and ESCC tissues recently[5-8].

Two-dimensional gel electrophoresis has been already studied in Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran to compare the protein populations of normal esophagus and ESCC tissues[9]. A predominant transition of C to T at CpG dinucleotides has been reported for p53 gene as well as over expression of cox-2 gene and accumulation of nitrotyrosine was detected in ESCC tumors of Iranian patients[10] confirming the concept of chronic inflammatory stress and sensitivity of esophageal cells to exogenous risk factors are involved in ESCC development in Iranian patients.

In order to find new molecular markers suitable for diagnosis and to identify probable potent molecular targets for drug design we started to screen genes with different levels of expression in normal and ESCC tissues of patients that were operated in 2 000 in Madean Hospital. Using differential display methodology on fresh normal and cancer tissues in this propose we scanned expression of almost 6 000 genes. Using this

technique we tried to screen out all real mRNA population of transcribed genes in tumor and normal tissues in order to include the probable unknown genes too. Among them 97 cDNAs were found with lower or higher expression levels in comparing normal and tumor tissues. 47 cDNAs with remarkable different levels of expression between normal and tumor states were cloned. Six genes that confirmed to have different expression in normal and ESCC tumor tissues are reported here.

MATERIALS AND METHODS

Tissue collection and sample preparation

Tumor tissues of ESCC patients and normal adjacent parts were surgically removed and snap frozen in liquid nitrogen and stored at -80 ℃ until use. By histopathological examinations tumors were classified into three major groups of well, moderately, and poorly differentiated squamous cell carcinoma according to WHO classification by a pathologist. A brief pathological and clinical data of the patients were shown in Table 1[12]. Histological normal adjacent epithelium from the same patient was used as the normal pair. A pair of tumor and normal tissues both from the same patient (RP; as reference patient) was chosen randomly for differential display. According to the clinical date this patient is a 47-year-old woman carried moderately differentiated tumor with reactive lymph nodes, which is an indicative of non-metastatic tumor. For a survey of gene expression in other ECSS patients, northern blotting was conducted for RP and nine other ESCC patients at different stages of tumor development. Patients were middle- to old-age all living in north of Iran.

Table 1 Sex, age and histopathological status of the inspected ESCC patients

No	Sex	Age	Differentiation degree of ESCC tumor	Metastatic lymphnodes
1	F	47	Moderately differentiated	None
2	M	53	Well differentiated	3 out of 8
3	F	47	Well differentiated	2 out of 10
4	F	50	Moderately differentiated	1 out of 1
5	M	69	Moderately differentiated	3 out of 9
6	F	60	Moderately differentiated	1 out of 6
7	M	66	Moderately differentiated	All five
8	M	76	Moderately differentiated	None
9	F	55	Poorly differentiated	No evidence
10	F	70	Well differentiated	All three

RNA purification

Using SV Total RNA extraction Kit (Promega), total RNA was purified from ESCC tumor and normal tissue pairs of the same patient in parallel. After DNase-I treatment, DNA-free RNA was recovered in a solution of 0.01 mol/L dithiotriotol (DTT), 10 U/L RNasin (Promega) in diethyl pyrocarbonate-treated water. The quality of RNAs and quantifications were done by spectrophotometry of Absorbency at 260 nm and gel electrophoresis.

Differential display reverse transcriptase-PCR

After 5 min of incubation of 0.2 g of RNA samples at 65 ℃, reverse transcriptions were done in 20 L reactions RT buffer containing 50 mmol/L Tris-HCl (pH 8.3), 125 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.01 mol/L DDT, 0.2 mol/L of a anchored oligo-dT11M (where M is A, C or G), 20 mol/L dNTPs and 200 U SuperScript™ RNase-H$^-$ Reverse Transcriptase (Gibco BRL), for 60 min at 37 ℃. For amplification 2.0 μL of each reverse transcribed RNA was added into a 20 l reaction containing 1.25 mmol/L $MgCl_2$, 2.0 μmol/L dNTPs, 0.2 μmol/L of the respective oligo-dT 11 mol/L, 0.2 mol/L of one 13-mer arbitrary primer, 1 μL [^{35}S]dATP [4.44×10^4 GBq/mmoL (1 200 Ci/mmoL), Amersham] and 1.0 U of Ampli-Taq (Perkin Elmer). PCR cycles/parameters were: a denaturation step at 94 ℃ for 5 min followed by 3 cycles at 94 ℃ for 30 s, 42 ℃ for 2 min, 72 ℃ for 30 s, and 37 cycles at 94 ℃ for 30 s, 45 ℃ for 2 min, 72 ℃ 30 s and a final extension at 72 ℃ for 5 min.

Identification and cloning of differentially expressed cDNA bands

The amplified products of DDRT-PCR were fractionated on standard 60 g/L poly-acrylamide/urea gel, and then transferred to 3 mm filter paper (Amersham) without methanol/acetic acid fixing, dried and exposed to X-ray film overnight. The putative differentially displayed bands that showed the same pattern of expression in repeated DDRT gels were eluted and re-amplified by the same primers used in related first amplification sets. Molecular mass and quality of re-amplified cDNA fragments were checked by gel electrophoresis before cloning into the cloning vector pCR2.1 (Invitrogen Co, CA). Escherichia coli TOP10 harboring recombinant plasmids of pCR2.1 were selected on agar plates containing ampicillin and X-gal. Since probable heterogeneity in cDNA populations of the same size existing in each band can leads to false positive results, for each cloned band six single well grown colonies were subjected to Southern blot. Radio-labeled probe was prepared from the inserted fragment of one of these six colonies.

Slot and northern blots analysis

For slot blots preparation, 2.0 g of RNA samples were vacuum-dried and resuspended in 50 L of DEPC-dd H_2O. Fifty microliters of formaldehyde/20SSC solution (1:1 vol/vol) was added and incubated for15 min at 60 ℃ before loading on nylon filters (Hybond, Amersham, UK). For northern blots, 30 g of N and T total RNAs were run on denaturing 1.2 g/L agarose gel and blotted onto Hybond membrane dried at 37 ℃ and fixed using UV cross-linking. As probes, gel purified cDNA fragments of interest were radio-labeled using High Prim™ DNA labeling system (Boheringer) and 4 of [-^{32}P]-dCTP [1.11×10^5 GBq/mmoL (3 000 Ci/mmoL), Amersham]. Blots were hybridized with respective probes in hybridization solution containing 0.5 mol/L phosphate buffer pH 7.2, 10 mmol/L EDTA and 50 g/L SDS at 60 ℃ overnight. Filters were washed twice at 55 ℃ in 0.1 hybridization solution for 15 min and scanned with a phosphor imager SF (Molecular Dynamics) following 4 h exposure at room temperature. Differentially expressed cDNA clones were sequenced using Sequenase kit Version 2.0 (USB) and fractionated on standard sequencing gels[13].

Analysis of the sequences

Data entry, sequence management, sequence alignment and protein sequence analysis were performed by Lasergene software package[12]. Sequence similarity search and several structural features were predicted by the use of online databases and related software including BLAST N, X and P (www.ncbi.nlm.nih.gov/BLAST/)[13,14], Pfam (www.sanger.ac.uk/Software/Pfam/search.shtml)[15], PRINTS (www.bioinf.man.ac.uk/dbbrowser/PRINTS/)[16,17], Blocks (condor.urbb.jussieu.fr/logiciels/Protein_Blocks/Structural_Words/motif_PB_m.html)[18], SMART (www.bork.embl-heidelberg.de/NAIL/RSmart)[19,20], PROSITE (www.expasy.org/prosite/)[21] and PSORT (psort.nibb.ac.jp/form.html)[22].

RESULTS

Isolation of differentially expressed genes

The expression pattern of about 6 000 genes was scanned using combinations of 24 arbitrary 13-mer primers and three different anchoring oligo-dT primers in differential display reverse transcription-PCR reactions. We identified 97 genes with

different levels of cDNA in tumor and normal tissues of esophagus. 47 bands with considerable differential level of cDNA signals in tumor and normal tissues (Figure 1 panel A; Table 2) were reamplified and cloned. The clones that confirmed differential expression by slot blot (Figure 1 panel B) were selected for sequencing and northern analysis on other tumor samples. To annotate the isolated cDNA fragments, corresponding full-length gene sequences was predicted by the use of human genome project data (Table 2). The generated transcripts were translated and used for various protein motif searches.

Down regulation of mal gene

A 360-bp cDNA fragment, ESC1 clone, was found down regulated in ESCC tumor cells (Figure 1). ESC1 sequence was localized to 2cen-q13 locus of chromosome 2 that encompass mal gene, a T-cell differentiation antigen. The amino acid sequence encoded by ESC1 aligned with the last 34 amino acids at the C-terminal of human MAL protein family members (Table 2). The available data shows that mal gene encodes four transcript variants as a result of alternative splicing. The detected band with estimated mRNA size of almost 1.1 kb by northern blot, strongly suggests that Mal-a variant is the only form expressed in normal esophagus. The expression of this gene was found to be down regulated in all 10 surveyed patients carrying tumors at different stages of ESCC (Figure 2).

Induced expression of a member of ras oncogene family

Another under-represented gene was found as a 273-bp cDNA fragment named ESC2 (Figure 1). The expression level of the related gene to ESC2, with approximate length of 1.0 kb mRNA,

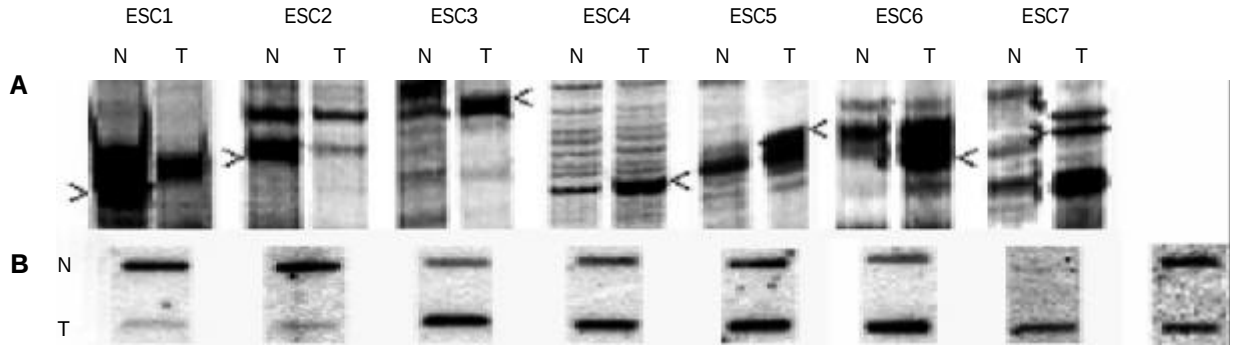

Figure 1 Parts of DDRT gels including cDNA fragments that show transcriptional alteration in comparing normal esophageal and ESCC tissues (have been pointed with arrows (panel A)), and the results of slot blots containing two microgram RNAs of normal (N) and tumor (T) tissues of esophagus in hybridization with radio-labeled related cDNAs (panel B). All of the slots have been prepared simultaneously and from the same RNA stocks of T and N. One piece of the slots has been hybridized with radio-labeled GADPH probe as shown in the right most side in panel B.

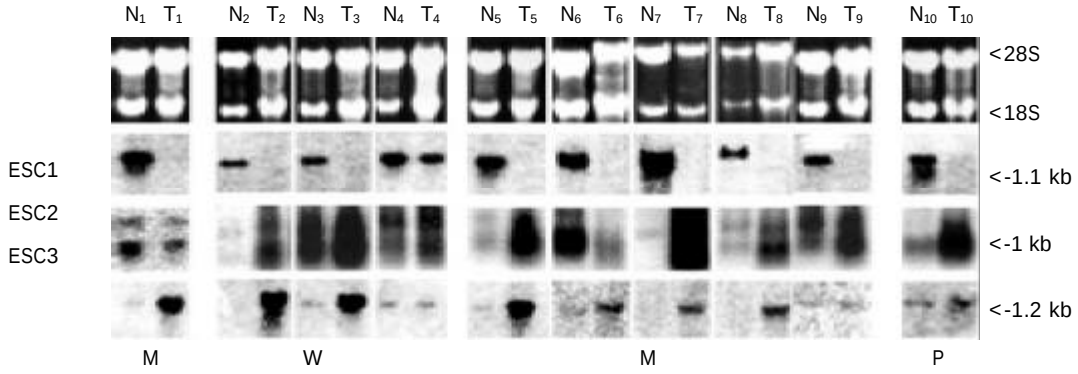

Figure 2 Northern results of patient 1 (RP) and other patients in hybridization with radio-labeled probes of ESC1, ESC2 and ESC3. Each northern membrane contains about 30 micrograms RNA of normal (N) and tumor (T) tissues of esophagus. The differentiation status of each tumor has been shown as W: Well differentiated tumor; M: Moderately differentiated tumor; P: poorly differentiated tumor. The estimated sizes of each bands has been shown on the right side and the ethidium bromide stained gel on top shows the relative amounts of RNAs loaded in each lane.

Table 2 The cDNAs, primers have been used in RT reactions and their related genes with different expression level in normal esophageal and ESCC tissues

cDNA name	cDNA's length (bp)	3' end primer (5'→3')	5' end primer (5'→3')	GeneBank accession number
ESC1	360	AAG CTT TTT TTT TTT G	AAG CTT TAC GTA C	BC000458
ESC2	273	AAG CTT TTT TTT TTT A	AAG CTT CTC AGA C	BC013348
ESC3	300	AAG CTT TTT TTT TTT C	AAG CTT CTC AGA C	BC007024
ESC4	325	AAG CTT TTT TTT TTT C	AAG CTT AAC GAGG	BC006095
ESC5	442	AAG CTT TTT TTT TTT G	AAG CTT GAT TCG C	BC000072
ESC6	271	AAG CTT TTT TTT TTT C	AAG CTT GCG ATG T	AF_061732
ESC7	273	AAG CTT TTT TTT TTT C	AAG CTT TGG TCA G	NM001759

in RP and patient no. 6 was consistent with that observation. However it showed increased level of expression in five tumors (no. 2, 5, 7, 9, 10) out of eight other patients (Figure 2). The sequence of ESC2 was identical to a sequence localized at chromosome 15q21 of human genome corresponding to the 3' region of human rab11a gene. The expression of this gene was down regulated in RP and patient no.6 while over-expressed in 5 other surveyed patients and unchanged in three others. No clear correlation was detected between the rab11a expression and pathological parameters and/or patients prognosis.

Up-regulation of an aldo-keto reductase encoding gene

A 300 bp of cDNA fragment, ESC3 clone, was clearly up regulated in ESCC tumor (Figure 1). ESC3 sequence identified a locus on chromosome 10 p15 encoding AKR1C1, 2 and 3 three members of aldo-keto reductase protein family 1C. The nucleotide sequence of ESC3 is identical to 3' end of AKR1C2. The corresponding protein sequence encompasses all six conserved blocks of the family. This gene is highly expressed in 8 out of 10 tumors of different stages of ESCC (Figure 2). Low abundance of its transcripts was detected in the other two patients. Again, no clear correlation was found between the expression level and histopathological classification of tumor samples (Figure 2) (Table 2).

Other differentially expressed genes

The expression of two cDNA fragments ESC4 and 5 with the length of 325 bp and 442 bp, respectively, were found to be induced in tumor tissues (Figure 1). They are localized on locus 5q32, the location of ribosomal protein L7 (RpL7), which is a member of RPL30 gene family, and on chromosome 19q13.4 that juxtaexpose the location of ribosomal protein 28 (RpL28) encoding gene respectively. Both of these ribosomal proteins are the components of 60S subunit of ribosome. Northern blots showed increase expression of RpL7 (ESC4) in three tumor samples of ESCC (pair no.1; 4 and 5 if RNA shown in the first panel is normalized), while there is no substatial alterations for its expression in other pairs (Figure 3). The over-expression of RpL28 (ESC5) was confirmed on at least 4 out of 6 ESCC tumor samples by northern (Figure 3, pair samples No: 1, 3, 5 and 8). ESC6 clone carries a 271 bp cDNA fragment that contains a sequence identical to100-amino acid hypothetical protein, which is moderately over expressed in tumor tissues of RP. A longer transcript of this sequence is also seen in related counting constructed from ESTs in TIGR and GeneBank (Table 2). This contig for ESC6 locates on 19p13.2 as shown by BLAST searches in Map Viewer. This aligned with a fetus brain protein called My029. The motif searches in BLOCKS, Pfam, CD, TIGR fam failed to identify any conserved motif, while there are two bipartite nuclear targeting sequence (RRqpslrglksrrkprc), three protein kinase C phosphorylation sites as showed by PSORT seraches. Northern analysis shows a moderate over expression of this gene in at least 4 out of 6 samples of well and moderately differentiated ESCC tumors (Figure 3).

The sequence of a 273 bp cDNA fragment, ESC7, localized in 12p13 that code for cyclin D2. Northern analysis on normal and tumor RNA of RP indicates the over expression of this gene in ESCC (Figure 1). No further work was done on this clone.

DISCUSSION

The expression level of two genes encoding MAL, and AKR1C2 have been enormously altered, so that they may be mentioned as switched off and on genes respectively, while the other genes as ribosomal proteins L7 and L28 and a hypothetical protein showed moderate differences of expression between normal and tumor tissues. Except for mal gene[23], differential expression of the other genes in ESCC is reported for the first time in this study.

ESC1 and 2 are the related clones for genes encode for two membrane proteins MAL and Rab11a respectively. A clear suppression of mal gene is seen in all ten ESCC samples (Figure 2). Mal gene has been already shown to express in four alternatively spliced forms of transcripts during intermediate and late stages of T-cell differentiation[24], also in differentiating epithelial cells[25]. The possible roles for MAL¨ Cglycosphingolipid complexes in cell signaling, differentiation and apical sorting have been suggested[25]. Here, we report down-regulation of this gene in all surveyed Iranian ESCC patients with different stages of tumor development, which is in good agreement by similar observations from China[23] and Japan[26]. As mal gene is up-regulated during the late stages of T-cell[24] and urothelial cells[25] differentiation, the switching-off of this gene in ESCC may suggest its involvement in a determining event in developing esophageal cancer. Although further studies are needed to clarify the role of MAL in normal esophageal cells and regulation of its expression, in a recent study the ectopic expression of MAL in carcinoma TE3 cells led to repressed formation of tumor induced by TE3 cells in nude mice, inhibition of cell motility and production of apoptosis by Fas pathway[26]. These observations suggest MAL protein as a potential candidate for ESCC diagnosis/ treatment and we expect it to be a tumor suppressor protein as it has been reported just recently[26].

Rab11a, a non-oncogene member of small GTPase Ras oncogene family, involves in secretory pathways, vesicle trafficking and apical recycling system in epithelial cells[27]. The most important role of Rab11a might be to facilitate cell migration

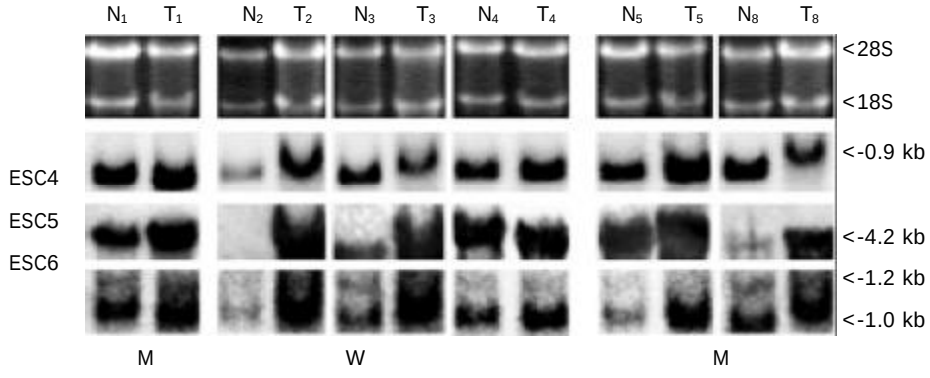

Figure 3 Northern results of patient 1 (RP) and other patients in hybridization with radio-labeled probes of ESC4, ESC5 and ESC6. Each northern membrane contains about 20 µg RNA of normal (N) and tumor (T) tissues of esophagus. The differentiation status of each tumor has been shown as W: Well differentiated tumor; M: Moderately differentiated tumor. The estimated sizes of each bands has been shown on the right side and the ethidium bromide stained gel on top shows the relative amounts of RNAs loaded in each lane.

by internalizing integrins at the rear of cell and transporting them forward at the leading edge to form new contacts with the extra cellular matrix[28]. To our knowledge, this is the first report of alterations of Rab11a expression in ESCC cancer. Similarly, massive synthesis of mRNA and protein of Rab11 was observed in cell line of esophagus adenocarcinoma and in low-grade dysplastic cells during esophageal adenocarcinoma development[29]. Roles rab11a can play in ESCC is to be studied.

One of the most important results of this study was demonstration of the up regulation of gene encoding AKR1C2 that catalyzes the interconversion of aldehydes and ketones to their corresponding alcohol compounds in sex hormones and similar substrates. Such reactions lead to the production of reactive oxygen species (ROS) and consequent DNA damage as carcinogenesis progresses[30]. Akr1c2 is also over expressed in lung carcinoma cell line[31], in non-small cell lung cancer[32], and in ovarian carcinoma cells[33]. The pertinent enzyme activity, dehydration dehydrogenase type 2 (DDH2), (EC 1.1.1.213) plays a critical role in metabolism of steroid hormone[34], drugs[35] and polycyclic aromatic hydrocarbons (PAH)[36]. As the esophagus tissue is not directly involved in steroid hormone metabolism the most probable substrate for this enzyme can be polycyclic aromatic hydrocarbons (PAH), some drugs and so on[35]. During metabolic pathway of these xenobiotic compounds, the resulting o-quinones lead to stable depurination of DNA adducts[36] that in turn can establish futile redox cycles and amplify ROS. These by-products are potent causative agents for establishing G to T transversion commonly found in ras and p53 genes[37,38] as shown to occur in ESCC tumors too[10]. Indeed oxidative stress has been considered as a major pathogenic factor in esophageal carcinoma[39].

Furthermore oxidative stress leads to 4-hydroxy-2-nonenal (HNE) formation during lipid peroxidation, which diffuses through the cell membranes and with a longer half-life, it is even more destructive than ROS[40]. On the other hand HNE specifically induces the expression of AKR1C1 isoform[40]. Since AKR1C2 and AKR1C1 differ for only seven amino acids, it is indistinguishable if AKR1C1 is also being over-expressed in ESCC. As these two enzymes differ just slightly in their untranslated 3' end regions, at least some portions of the signal in northern can belong to akr1c1 transcripts so that both enzymes may be up-regulated in ESCC.

A clear up-regulation of akr1c2 in 8 out of 10 tumor samples of Iranian ESCC patients with different degrees of tumor development was shown in Figure 2. This enzyme is suggested to be involved in esophageal carcinogenesis at least indirectly, and this speculation is in a good agreement with the hypothesis of PAH exposure association with carcinogenesis of this tissue[41]. However AKR1C2 is of special interest in cancer biology and drug design for prostate cancer[42] and may be considered also as a potent molecular target in ESCC treatments.

The expression of RpL7 is higher in tumor tissues of all three samples of ESCC, wherein the amount of normal and tumor RNA can be normalized, considering the signals showed in Figure 2 for ESC4. The up-regulation of RpL7 has been reported recently in prostate cancer too[43]. Several ribosomal proteins are suggested to be involved in cell proliferation, apoptosis, DNA repair, regulation of development and malignancies as their second function[44], however the possible role of this protein in cancer cells remains to be clear. ESC5 cDNA fragment, that identified as RpL28 shows a higher expression in tumor tissues of ESCC. RpL28 seems to have a regulatory role as it is strongly ubiquitinated during S phase in Saccharomyces cerevisiae, while the levels of RpL28 ubiquitination is reduced in G1. As the human ortholog of RpL28 is also ubiquitinated, this modification is highly conserved in evolution, and a probable regulatory role for multiubiquitin of RpL28 has been speculated not necessarily as a target for degradation[45].

We have seen also the over expression of cyclin D2 using Northern blot on RNA samples of RP, but no more experiments have done on other patients because of low amounts of available RNA. Although the over expression of Cyclin D2 in different kinds of squamous carcinoma cell lines has been shown to modulate proliferation after induced quiescence and enhance *in vivo* aggressive growth behavior[46], however the RP sample has no metstasis in pathology report of tumor.

It is obviously anticipated that many genes are involved in carcinogenesis of esophagus, although differential expression of these genes could be the consequence rather than a cause. Many complicated events like cell to cell/matrix interactions, cell communications and transportations, normal regulation of protein synthesis, translation and cell cycle regulation as well as metabolic enzymes are affected by cancer development of esophagus.

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