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• ESOPHAGEAL CANCER •

Expression patterns of esophageal cancer deregulated genes in C57BL/6J mouse embryogenesis

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Abstract

AIM: To investigate the expression patterns of esophageal squamous cell cancer deregulated genes in mid to late stages of C57BL/6J mouse embryogenesis, and the correlation between these genes in embryonic development and tumorigenesis of esophageal squamous cell cancer.

METHODS: Reverse northern screening was performed to examine the expression patterns of esophageal cancer deregulated genes in C57BL/6J mouse embryogenesis. To confirm the gene expression patterns, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out for 3 of the randomly picked differentially expressed genes.

RESULTS: Within these esophageal cancer deregulated genes, 4 patterns of expression were observed at 3 stages embryonic d 11.5 (E11.5), embryonic d 13.5 (E13.5) and postnatal d1 (P1). (1) Up-regulation during the E11.5 period, down- regulation during the E13.5 and P1 period (up-down-down), the 10 up-regulated genes during the E11.5 period could be classified into 6 known genes and 4 unknown genes. The known genes included differentiation related genes (S100A8), immunity related gene (IGL), translation and transcription regulation genes (RPL15, EEF1A1), cytoskeletal protein (TUBA1), cysteine protease inhibitor (cystatin B). (2) Up-regulation during the E13.5 and P1 period (down-up-up), such as the SPRR2A which was down-regulated at E11.5. (3) Down-regulation during the E11.5 and E13.5 period (down-down-up), such as RHCG and keratin 4. (4) Fluctuating expression, down initially, up at E13.5, and then down again (down-up-down). EMP1 belonged to such a gene, which was highly expressed at E13.5.

CONCLUSION: The results will be helpful for understanding the function of esophageal squamous cell carcinoma (ESCC) deregulated genes in embryonic development and tumorigenesis. S100A8 and S100A9 may play different roles in early embryonic development. IGL may be an oncofetal protein, and EMP1 relates with neurogenesis at E13.5. The genes identified pertinent to embryonic development may serve as candidate susceptibility genes for inherited esophageal cancer disorders as well as for various heritable disorders of embryonic development.

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INTRODUCTION

Esophageal cancer is one of the most lethal malignancies in the world. It exists in 2 main pathological types, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EADC). ESCC is the predominant histological subtype of esophageal cancer and characterized by high mortality rate and regional variation in incidence in China[1]. Due to the relatively late stage of diagnosis and poor treatment, its five-year survival rate remains below 10%[2]. The development of better treatment modalities and better diagnostic and preventive approaches requires a understanding of the molecular mechanisms of the complex process of esophageal tumorigenesis.

Tumor development involves up-regulation and down-regulation of many genes. The study of these genes is important to understand the complex biological events that take place during the malignant transformation of normal tissues. cDNA microarray technology allows simultaneous determination of the expression level of thousands of genes[3]. Up to date, by using the cDNA array technology, we have identified many genes differentially expressed in esophageal cancer, which potentially play key roles in the malignant behavior of esophagus[4,5]. Investigation into the biological function of these genes could be helpful to understand the molecular mechanisms in tumorigenesis of ESCC.

Mouse is an ideal model system for studying the molecular mechanisms underlying the pathogenesis of human cancer. The generation of transgenic and gene-knockout mice has been instrument in determining the role of major determinants in this process, such as oncogenes and tumor-suppressor genes. In addition, mice at different development levels also provide a useful model for functional study of the genes. Understanding the temporal and spatial expression of a gene in embryogenesis will be useful for further analysis of its function in cancer[6,7]. Here, the expression patterns of some ESCC deregulated genes in 3 embryonic stages were analyzed using reverse northern screening. Selected genes were further confirmed by RT-PCR assays. As a result, we found that genes with specific developmental functions were expressed at specific developmental stages. These genes cover a broad spectrum of cell biology processes including cell growth, differentiation, matrix metabolism, immunity response, and would be of significance to understanding the tumorigenesis of esophagus.

MATERIALS AND METHODS

Animal and tissue specimens

Time-mated C57BL/6J mice were obtained from Animal Institute, Chinese Academy of Medical Sciences. Mice were obtained from in-house breeding programs and maintained on a 12:12-h light-dark cycle and provided standard laboratory food and water. All manipulations of mice were done in accordance with policies of the Institute Animal Care and Use Committee. Timed mating was set up between adult wild-type mice. Females were inspected for plugs on the following day to ensure their successful mating. The day of plug detection was considered as E0.5. Embryo was dissected from pregnant mouse at the age of embryonic d 11.5 (E11.5), 13.5 (E13.5), and postnatal d 1 (P1). Three samples per time point were analyzed for statistical significance. The tissues were immediately frozen in liquid nitrogen until analysis.

Total RNA extraction

Total RNA was extracted from frozen tissues by using TRIzol™ reagent following the protocols of the manufacturer (GIBCO/BRL, New York, USA). The quality of RNA was confirmed on a formaldehyde agarose gel, and concentration was determined by reading the absorbance at 260/280 nm.

cDNA fragment acquisition

Plasmids of interested clones were selected from esophageal cDNA libraries and used as PCR templates. The primers used were T3 (AAT TAA CCC TCA CTA AAG GG) and T7 (GTA ATA CGA CTC ACT ATA GGG C). The average length of cDNA fragments was about 1 kb. The homology of these genes between human and mice was more than 50%.

Reverse northern screening

PCR fragments corresponding to 90 distinct clones were diluted in 0.5 mol/L NaOH and 1.5 mol/L NaCl and 0.2 mL (approximately 40 ng) of every slim was transferred onto duplicate nylon membranes (HyBond-N, Amersham Pharmacia Biotech, Arling-ton Height, IL). The filter membranes were neutralized with 1.5 mol/L NaCl and 0.5 mol/L Tris-HCl (pH 7.5) for 5 min, dried at 80 ℃ for 60 min in the oven. cDNAs corresponding to the housekeeping gene β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls.

To generate the probes, ^{32}P-labeled cDNA was synthesized from RNA derived from mice of different embryonic period. mRNA was incubated with random hexamers (Promega Corp.) for 5 min at 65 ℃, chilled on ice for at least 1 min, and added to a solution containing 50 U superscript IIRT in 20 mmol/L Tris-HCl (pH 8.4); 5 mmol/L $MgCl_2$; 0.5 mmol/L each of deoxy (d)-ATP, dTTP, and dGTP, 5 mmol/L dCTP; and 50 mCi [^{32}P] dCTP in a final volume of 20 μL. After 50 min of incubation at 42 ℃, followed by incubation at 70 ℃ for 15 min, parental mRNA was removed from the cDNA synthesis reaction by addition of RNase H and incubation for 20 min at 37 ℃. Unincorporated nucleotides were removed by gel filtration on Sephadex G-25 preloaded spin columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The probe was denatured at 90 ℃ for 5 min before added to the hybridization solution.

The filter membranes were washed in 6×SSC and pre-hybridized for 5 h at 68 ℃ in a buffer containing 6×SSC, 2×Denhardt's (1 g/L each of Ficoll 400, polyvinylpyrrolidone, and BSA), 1 g/LSDS, and 0.1 g/L denatured salmon sperm DNA. Hybridization was carried out for 18 h at 68 ℃ using ^{32}P-labeled cDNA probes added in fresh prehybridization buffer. After the hybridization, the filters were washed (2×SSC and 1 g/L SDS), then exposed to X-ray film at -70 ℃ overnight with an intensifying screen[8]. The images were scanned by Fluor-S MultiImager (Bio-Rad, California, USA) and the original intensity of every specific slim was quantitated with the software Multi-Analyst (Bio-Rad, California, USA). To avoid misinterpretation of the results possibly due to variation in the hybridization, any filter was normalized using 2 housekeeping genes, and a normalization coefficient calculated for each comparison was used to correct the signal intensities. The differential expression was considered as significant when the ratio of the signal from the same spot on different membranes was greater than 2.0.

Semi-quantitative RT-PCR

Total RNA was extracted by the method described above. Five micrograms total RNA of each sample was used to synthesize the first strand cDNA with SuperScript preamplification system for first strand cDNA synthesis kit (GIBCO/BRL, New York, USA). Then 1 μL RT product was used as the template to amplify specific fragments. PCR reaction conditions were optimized individually for each gene studied, and the cycle number for PCR was adjusted so that the reactions fell within the linear range of product amplification. The expression of housekeeping gene GAPDH, was used as an internal control. The RT-PCR reaction product was analyzed by electrophoresis on a 15 g/L agarose gel. Electrophoresis images were scanned by Fluor-S MultiImager (Bio-Rad, California, USA) and the original intensity of every specific band was quantitated with the software Multi-Analyst (Bio-Rad, California, USA). Data were compared after being normalized by the intensity of GAPDH. The sequences of PCR primers and cycle number are listed in Table 1.

Table 1 Primers used for semi-quantitative RT-PCR and conditions of PCR reaction

Gene	Dir	Primer sequences (5'-3')	Product size	Anne (℃)*	PCR cycles
S100A8	F	TGA CAA TGC CGT CTG AAC TG	271	60.4	28
	R	TCC TTG TGG CTG TCT TTG TG			
S100A9	F	CAG CAT AAC CAC CAT CAT CG	310	60.1	28
	R	TTA CTT CCC ACA GCC TTT GC			
EMP-1	F	AGT GCA AGC CTT CAT GAT CC	599	60.0	28
	R	TCC GAT CTG GGT CTC CAT AC			
CSTB	F	GTC CCA GCT TGA ATC GAA AG	208	59.9	28
	R	ATC GTG CCT TTC TTT GTT GG			
GAPDH	F	ACCACAGTCCATGCCATCAC	452	According to target gene	
	R	TCCACCACCCTGTTGCTGTA			

*F, forward; R, reverse; Dir, direction; Anne, annealing temperature.

RESULTS

Reverse northern data

The expression of esophageal cancer deregulated genes during C57BL/6J mouse embryogenesis showed a clear difference. As shown in Figure 1, no signals were visible in the negative control spots, indicating that the hybridization was highly specific. The housekeeping gene density was similar, indicating that the results were credible.

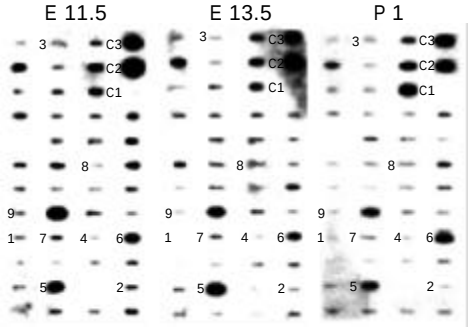

Figure 1 Representatives of esophageal cancer deregulated genes expressed in C57BL/6J mouse embryogenesis by reverse northern screening. Three filters were hybridized with ^{32}P-labeled cDNA probe from E11.5, E13.5 and P1 period. β-actin and GAPDH were used as controls. c1, negative control; c2, β-actin; c3, GAPDH; 1, S100A8; 2, IGL; 3, RPL15; 4, CSTB; 5, SPRR2A; 6, RHCG; 7, keratin 4; 8, EMP1; 9, S100A9.

By reverse northern screening, the results showed that the expression patterns of these genes were diverse and complex (Table 2). Four patterns of expression were seen at the 3 stages of E11.5, E13.5 and P1. (1) Up-regulation during the E11.5 period, down at E13.5 and P1 (up-down-down), the 10 up-regulated genes could be classed into 6 known genes and 4 unknown genes. The known genes included differentiation related genes (S100A8), immunity related gene (IGL), translation and transcription regulation genes (RPL15, EEF1A1), cytoskeleton protein (TUBA1), cysteine protease inhibitor (cystatin B). (2) Up-regulation during the E13.5 and postnatal d 1 period (down-up-up), such as the SPRR2A which was down-regulated at E11.5. (3) Down-regulation during the E11.5 and E13.5 period (down-down-up), such as RHCG and keratin 4. (4) Fluctuating expression, down initially, up at E13.5, and then down again (down-up-down), such as EMP1,which was highly expressed at E13.5.

Confirmation by RT-PCR

Three genes (S100A8, EMP-1, CSTB) with notable difference, as well as S100A9 were selected randomly for further confirmation by RT-PCR. As a result, the expression of these genes was consistent with the reverse Northern blot results (Figure 2). It indicated that the results of reverse Northern blot analysis were reliable.

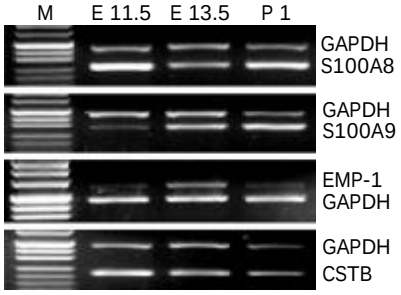

Figure 2 Expression patterns of several genes confirmed by multiplex semi-quantitative RT-PCR. All RT-PCR products were analyzed by electrophoresis on 15 g/L agarose gel. The expression of GAPDH was used as internal control and the size of GAPDH gene amplification was 452 bp.

DISCUSSION

Cancer and development are conceptually related. The normal embryonic development process might display many of the properties associated with tumor development and progression, such as rapid proliferation, migration of cells and formation of new blood vessels[9]. Tumor formation in many cases results from the aberrant expression of a developmental program. Events in the tumor have their normal regulatory counterparts in the embryonic development. Thus, research into the molecular change involved in development is important not only for understanding of embryogenesis, but also for understanding and managing cancer. In this study, using the C57BL/6J mouse as a model system, we investigated the expression pattern of esophageal cancer deregulated genes

Table 2 Expression pattern of esophageal cancer deregulated genes during C57BL/6J mouse development

Gene name	Unigene	E11.5	E13.5	P1	Expression pattern	Change in ESCC
S100A8	Hs.100000	0.12	0.06	0.05	up-down-down	down
IGL	Hs.181125	0.22	0.09	0.08	up-down-down	up
RPL15	Hs.74267	0.23	0.08	0.07	up-down-down	down
EEF1A1	Hs.181165	3.50	0.72	0.43	up-down-down	down
TUBA1	Hs.75318	9.06	1.13	0.54	up-down-down	down
cystatin B	Hs. 695	0.19	0.05	0.07	up-down-down	down
ASN 120	Hs.239758	5.04	0.99	1.00	up-down-down	down
ASN 141	Hs.15087	2.56	0.74	0.79	up-down-down	down
ASN 409	Hs.238513	2.86	0.3	0.34	up-down-down	down
ASN 109	Hs.154390	2.52	0.61	0.59	up-down-down	down
SPRR2A	Hs.355542	0.24	0.48	0.67	down-up-up	down
RHCG	Hs.279682	0.19	0.11	0.78	down-down-up	down
keratin 4	Hs.3235	0.08	0.05	0.35	down-down-up	down
EMP1	Hs.79368	0.16	0.32	0.16	down-up-down	down

IGL, immunoglobulin lambda locus; RPL15, ribosomal protein L15; EEF1A1, eukaryotic translation elongation factor 1 alpha1; TUBA1, tubulin alpha1 (testis specific); ASN, automated sequencing number; SPRR2A, small proline-rich protein 2A; RHCG, Rh type C glycoprotein; EMP1, epithelial membrane protein 1.

during 3 stages of embryogenesis. Esophageal cancer deregulated genes showed several kinds of expression pattern during embryogenesis, which provided useful information on the tumorigenesis of esophageal cancer.

During embryonic development of mice, genes exhibit complex patterns of spatial and temporal expression and direct organ development in a variety of ways. Differentially expressed genes in embryogenesis implicated that they might play important roles in development. Cancer cells resemble embryonic cells morphologically and share some characteristics with each other, such as rapid proliferation, reduced differentiation, and increased motility. The same intercellular signals that control proliferation and differentiation in development can control tumor development. These common signals are the focus of our study. In the present study, many esophageal cancer deregulated genes showed differential expression during mice embryonic development. These genes covered a broad spectrum of biologic functions including cell proliferation (RPL15)[10,11], differentiation (S100A8[5], SPRR2A[12]), immune response (IGL)[13], cytoskeletal protein (TUBA1)[14] migration (cystatin B)[15], *etc*. These findings provided further clues to the possible function of these deregulated genes and the tumorigenesis of ESCC.

The transition from cell proliferation to differentiation in embryonic development is under strict regulation. Cancer has been called a "developmental disorder"[16] because it involves a disruption of the normal developmental program for cells, in terms of both differentiation and proliferation. Esophageal cancer is always characterized by uncontrolled proliferation of epithelial cells and failure to differentiate to the normal phenotype. The genes controlling proliferation and differentiation and their transition are very important. In ESCC, S100A8 and S100A9 are both significantly down-regulated and contribute to tumor progression but their function is not fully understood. It has been reported that S100A8 and S100A9 can form a heterodimer and co-express in neutrophils, monocytes, and some secretory epithelia[17-19]. However, in this study, we found that the expression patterns of 2 members of S100 genes were not completely parallel during the process of embryogenesis. The expression of S100A8 was significantly higher at E11.5 than that of S100A9, but in the periods of E13.5 and P1 the 2 genes' expression patterns were similar. Previous studies have shown that S100A8 mRNA expressed without S100A9 mRNA within fetal cells infiltrating the decidua in the vicinity of the ectoplacental cone between 6.5 and 8.5 d postcoitum[20]. Targeted disruption of the S100A8 gene caused rapid and synchronous embryo resorption by d 9.5 of development in homozygous null embryos, but the S100A9-deficient mice were viable, fertile, and generally healthy[21]. These findings, together with the literature, suggest that S100A8 and S100A9 may play different roles in the embryonic development and process of ESCC.

The proliferative changes in cancer cells are usually accompanied with other changes in cellular activities, including reversion to a less differentiated, more developmentally primitive state. Genes expressed in embryogenesis, down-regulated with tissue maturation and re-expressed in cancer, are designated as oncofetal genes, many of which are used as tumor markers. In this study, we found that IGL was abundantly expressed at E11.5 of fetal life and down-regulated subsequently. Meanwhile, multiple myeloma (MM) related to immunoglobulin (Ig) types and light chain subtypes[22], research proved that kappa and lambda type immunoglobulin light chains could increase the percentage of viable polymorphonuclear leucocytes by inhibiting apoptosis in a concentration-dependent manner[23]. These findings, indicate that IGL may be an oncofetal protein, but the mechanism of IGL overexpression in ESCC is not clear. Its potential usage as a marker of early recurrence and in gene therapy needs to be further studied. Structural gene is also important in embryogenesis and tumorigenesis. TUBA1 had the largest expression change of all the genes at E11.5, 6.7-fold. As we know[14], microtubules are composed of tubulin alpha and beta. They participate in a large number of intracellular events including cell division, intracellular transport and secretion, axonal transport, and maintenance of cell morphology. The diminution of interphase cytoplasmic microtubules in tumor cells was probably due to the deficiency of microtubule organizing function in interphase tumor cells[24].

Gene expression in specific phase implicates its specific function in that period. In our study, we found that epithelial membrane protein 1(EMP1) was characteristically expressed at E13.5. It has been found EMP1 is a member of the PMP22 (peripheral myelin protein 22) family[25,26]. It was expressed in most tissues of the adult mouse, with highest levels in the gastrointestinal tract and lung[27]. High-level expression of EMP1 was associated with differentiation and growth arrest in squamous cells and the haematopoietic system[28]. At the molecular level, EMP1 was thought to be involved in the regulation of the cell cycle, cell-cell recognition, and cell death[29]. At E13.5, high EMP1 expression was found in the initial fiber tracts of the developing cerebellum[30]. In the second stage of embryonic brain development (E13 to E16), the neuroepithelium remained active, but was coupled with the differentiation of neurons. Hence, the observed increased expression of EMP1 at E13.5 during the second stage of prenatal brain development, is related with the potential function of this molecule during neurogenesis. Whether the absence of EMP1 at P1 and E11.5 is due to down-regulation of expression in maturing cells or EMP1 expression is restricted to some embryonic tissue remains to be elucidated.

In summary, the results from the present study can contribute to our understanding of the function of deregulated genes in ESCC, which are important in both embryonic development and tumorigenesis. These genes identified in the special developmental stage of mice may play some roles in the control of growth, terminal differentiation and may have physiologic or pathological importance during normal development process and the development of ESCC. Finally, the genes identified pertinent to embryonic development may serve as candidate susceptibility genes for inherited esophageal cancer disorders as well as various heritable disorders of embryonic development. Further study is required for the precise relationship between the altered genes and the pathogenesis of esophageal squamous cell cancer and embryonic development.

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