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• ESOPHAGEAL CANCER •

Using yeast two-hybrid system to identify ECRG2 associated proteins and their possible interactions with ECRG2 gene

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Abstract

AIM: To identify esophageal cancer related gene2 (ECRG2) associated proteins and their possible interactions with ECRG2 gene.

METHODS: In the yeast forward two-hybrid system, ECRG2 was fused with the DNA-binding domain (DBD) of Gal4 and human fetal liver cDNA library was fused with the transcriptional activation domain (AD) of Gal4. We performed a high-stringency scale procedure to screen ECRG2 against human fetal liver cDNA library and characterized positives by sequence analysis.

RESULTS: We found the following 9 putatively associated proteins. They were metallothionein2A, metallothionein1H, metallothionein1G, ferritin, erythrocyte membrane protein band4.2, mitochondrial ribosomal protein S12, hypothetical protein FLJ10101, and a novel gene whose cDNA was found to have no strong homology to any other previously characterized gene whose DDBJ/EMBL/GenBank accession number is AF422192 mapped to human chromosome 14q31.

CONCLUSION: MT, a potential interaction partner for ECRG2, might be involved in the regulation of cell proliferation and apoptosis, and in various physiological processes. Determination of a reliability score for each single protein-protein interaction, especially interaction of ECRG2 and MT, permits the assignment of ECRG2 and unannotated proteins to biological pathways. A further understanding of the association between ECRG2 and MT should facilitate the functions of ECRG2 gene.

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INTRODUCTION

Esophageal cancer (EC) is one of the most common malignancies worldwide with the highest mortality and prevalencet in certain areas of China with a higher incidence[1-2]. It has been found some of tumor suppressor genes and oncogenes are involved in the EC initiation and development[3, 4]. However, so far no gene directly related to EC has been identified. By comparing the differential gene expression between normal esophageal epithelia and esophageal cancer using the technique of mRNA differential display, we have cloned a new gene ECRG2 (GenBank Accession No. AF268198) whose expression was found to be down-regulated in some malignant tissues such as esophageal carcinoma tissue, colon cancer and brain tumor tissues, and most common in esophageal cancer tissues. Although SMART online (http://www.smart.embl-heidelberg.de) has shown that ECRG2 gene contains a characteristic KAZAL-type conserved domain and belongs to the KAZAL-type related serine proteinase inhibitor family, little has been known about its function in normal cellur activities, other than suppressing neoplasia. In order to further reveal its biological roles, we therefore tried to identify ECRG2 associated proteins in the present study by the GAL4-based yeast two-hybrid system using the full-length ECRG2 cDNA as a bait to screen the human fetal liver cDNA library.

MATERIALS AND METHODS

Yeast strains

Matchmaker GAL4 two-hybrid system 3 and vector pACT2 containing the human fetal liver cDNA library were obtained from Clontech. Yeast strain AH109 (MATa, trp 1-901, leu2-3, 112, ura 3-52, his3-200, gal4△, gal80△, LYS2: $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3: $MEL1_{UAS}$-$MEL1_{TATA}$-LacZ) was used to screen the library and to verify protein-protein interactions, which could eliminate false positives by using three reporters-ADE2, HIS3, and MEL1 (or LacZ)-under the control of GAL4 upstream activating sequences (UASs) and TATA boxes. Among these reporters ADE2 provided strong nutrition selection, HIS3 gave a selection reducing false positive incidence, and MEL1 or LacZ encoding β-galactosidase could be assayed on X-α-gal indicator plates.

Plasmid constructs

Vector pGBkT7 expressing proteins were fused with amino acids 1-147 of the GAL4 DNA binding domain (DNA-BD), pGADT7 expressing proteins were fused with amino acids 768-881 of the GAL4 activation domain (AD). The control plasmids pGBKT7-53, pGBKT7-lam, pGADT7-T, pCL1 were from Clontech. Plasmid pGBKT7-ECRG2 encoding full-length (85 amino acids) ECRG2 gene fused in frame with the GAL4 DNA binding domain was constructed by inserting the PCR-generated fragment into the EcoRI and BamHI sites of pGBKT7. Plasmid pGADT7-ECRG2 encoding full-length ECRG2 gene fused in frame with GAL4 activation domain was constructed by inserting the PCR-generated fragment into the EcoRI and BamHI sites of pGADT7.

Library tittering and amplification

For tittering library, an aliquot of the library plasmid was thawed and mixed on ice by gentle vortexing. Then 1μl of library plasmid was transferred to 1 ml of LB broth, which was mixed and named dilution A ($1:10^3$). By the same way,

another library plasmid diluent dilution B ($1:10^6$) was obtained. The prewarmed LB/amp plates were prepared by inoculating them in 1 μl of dilution A mixed with 50 μl of LB broth, or 50 μl and 100 μl of dilution B where respectively incubated on LB/amp plates at 30 ℃ for 48 h. The library titer was calculated as follows: Dilution A: counted colonies$\times10^3\times10^3$=cfu/ml, Dilution B: (counted colonies/plating volume)$\times10^3\times10^3\times10^3$ =cfu/ml. For the library amplification, LB/amp plates were inoculated with the library plasmids at such a high density that the resulting colonies could be easily confluent (-20 000-40 000 cfu per 150-mm plate) and reach at least 2-3 times as the library original plasmid number to ensure the better screening results. After incubated at 30 ℃ for 48 h, colonies were scraped, mixed with adequate volume of LB broth and shake-cultured in a flask at 30 ℃ for 2 h. One-third of the library culture was set aside for plasmid preparation.

Verification of activation of reporter gene by pGBKT7-ECRG2

Plasmid pGBKT7-ECRG2 was independently transformed into strain AH109. The transformants were assayed for β-galactosidase activity by selecting them on SD/-Trp/-Leu/X-α-Gal. Positive control, pCL1, was performed in parallel.

Library transformation and screening

Plasmid pGBkT7-ECRG2 was used as a bait in two-hybrid screening of human fetal liver cDNA libraries by MATCHMAKER Two-Hybrid System 3 protocol (Clontech). The yeast strain AH109 was sequencely transformed with pGBKT7-ECRG2 and a human fetal liver cDNA library in the pACT2 vector (Clontech) was obtained by the lithium acetate method. Transformants expressing both the bait and interacting prey proteins were selected on SD/-Trp/-Leu/-His/-Ade and incubated at 30 ℃ for 5-7 days. β-galactosidase activity was tested using the filter lift assay to identify the positive colones.

Isolating plasmid DNA from putatively positive yeast clones, rescuing AD/library plasmids and retesting protein interaction in yeast

Approximately 3×10^6 colonies were screened and 146 positive clones were identified. cDNA inserts of the positive clones were amplified by PCR using primers complementary to the sequence of pACT2 vector (5' T ACC ACT ACA ATG GAT3' and 5' GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG A3'). Subsequently, pACT2-cDNA constructs were isolated from positive yeast colonies, as recommended by the supplier, transformed into super-competent *E. coli* DH5α by electroporation, grown under selection, re-isolated and analyzed by restriction digests. The uniquely purified constructs were then re-tested against the original pGBKT7-ECRG2 bait construct. To ensure the interactions were specific, the positive clones were also tested against an irrelevant bait protein laminC and grown on SD/-Ade/-His/-Leu/-Trp/X-α-Gal to test the specificity of interactions.

Sequence Ad/library inserts and blast online (http://www.ncbi.nlm.nih.gov/blast)

The positive inserts were sequenced and analyzed by comparison to the GenBank sequence data bank.

RESULTS

Library tittering and amplification

The titer of human fetal liver cDNA library was 3.17×10^9cfu/ml counted by dilution method. The number of separate colonies was 1.05×10^7. We amplified it in 150 mm×500 plates, about 1×10^8 (3.5 μl original cDNA library) colonies and the library plasmids were successfully isolated.

Verification of activation reporter genes by pGBKT7-ECRG2

Plasmid pGBKT7-ECRG2 was independently transformed into AH109. After β-galactosidase activity assay, the AH109 transformed pGBKT7-ECRG2 did not appear blue colonies, but the positive control, pCL1, appeared as is shown in Figure 1. It was verified that pGBKT7-ECRG2 construct did not activate reporter genes and was suitable for the yeast two-hybrid system (Figure 1).

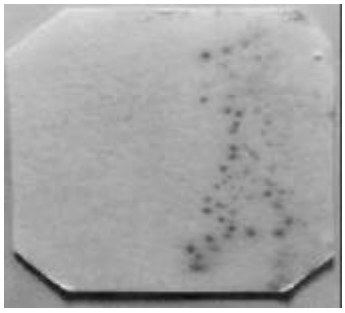


Figure 1 Colony-lift filter assay for β-galactosidase activity. Left: β-galactosidase activity of AH109 transformed with pGBKT7-ECRG2. Right: β-galactosidase activity of AH109 transformed with pCL1 (positive control). The results showed that pCL1 could activate reporter genes, but pGBKT7-ECRG2 could not.

Yeast two-hybrid screen of cellular proteins interacting with ECRG2 protein

To identify proteins associated with the ECRG2 protein, ECRG2's ORF was synthesized as a translational fusion of a DNA-binding domain (DBD) and used as the bait for screening of a human fetal liver yeast two-hybrid cDNA library. Of 3×10^6 transformants screened, 146 clones grew in the absence of tryptophan, lencine, histidine, adenine and expressed β-galactosidase activity. pACT2/cDNA plasmids were successfully isolated and duplicates were eliminated by HaeIII digestion (Figure 2). After elimination, 26 uniquely positive clones were further retested for specificity of β-galactosidase expression. After retransformation, 9 independent positive clones were identified and sequenced (Table 1).

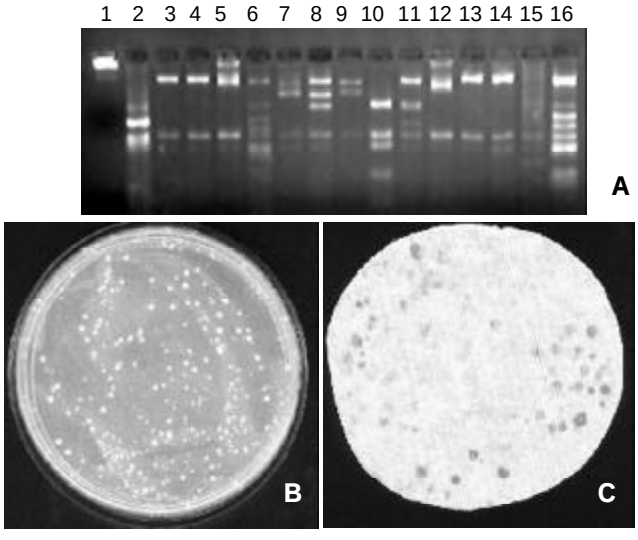


Figure 2 Positive colonies screened by yeast two-hybrid system using full-length cDNA of ECRG2 as baits. A: PCR products of positive clones digested with HaeIII restriction enzyme. Lane 1: λDNA/EcoRI+HindIII Marker, Lanes 2-16: positive colonies, B: $Trp^+/Leu^+/His^+/Ade^+$ positive clone growing on the $SD/Trp^-/Leu^-/His^-/Ade^-$ plate, C: colony-lift filter assay for β-galactosidase activity.

Table 1 Yeast two-hybrid screening using ECRG2 as baits

Yeast transformation	Transformation efficiency[a] (cfu / μg library)	Transformation yield[b]	His$^+$,Ade$^+$, Laz$^+$[c]	True positives
1	2.46×10^5	9×10^5	48	3
2	3.17×10^5	1.3×10^6	63	4
3	2.32×10^5	8×10^5	35	2
Total		3×10^6	146	9

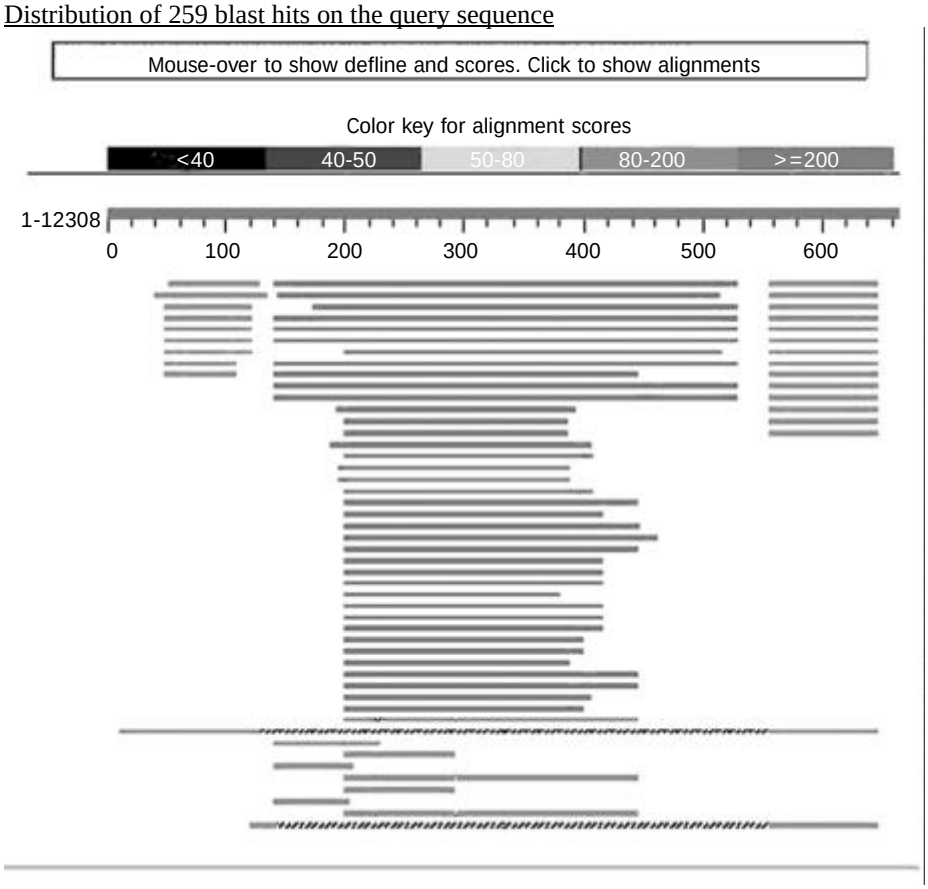
[a]: Transformation efficiency (transformants / μg) = transformation yield ÷ amount of library DNA in μg). [b]: Transformation yield (total transformants) = [(colonies / plate) ÷ (volume / plate)] ×[(volume of total reaction) ÷ (dilution factor)]. [c]: β-galactosidase activity of the positive colonies assayed by β-galactosidase filter lift assay.

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phases 0, 1 or 2 HTGS sequences)

1,431,609 sequences; 7,058,003,641 total letters

Taxonomy reports

Distribution of 259 blast hits on the query sequence



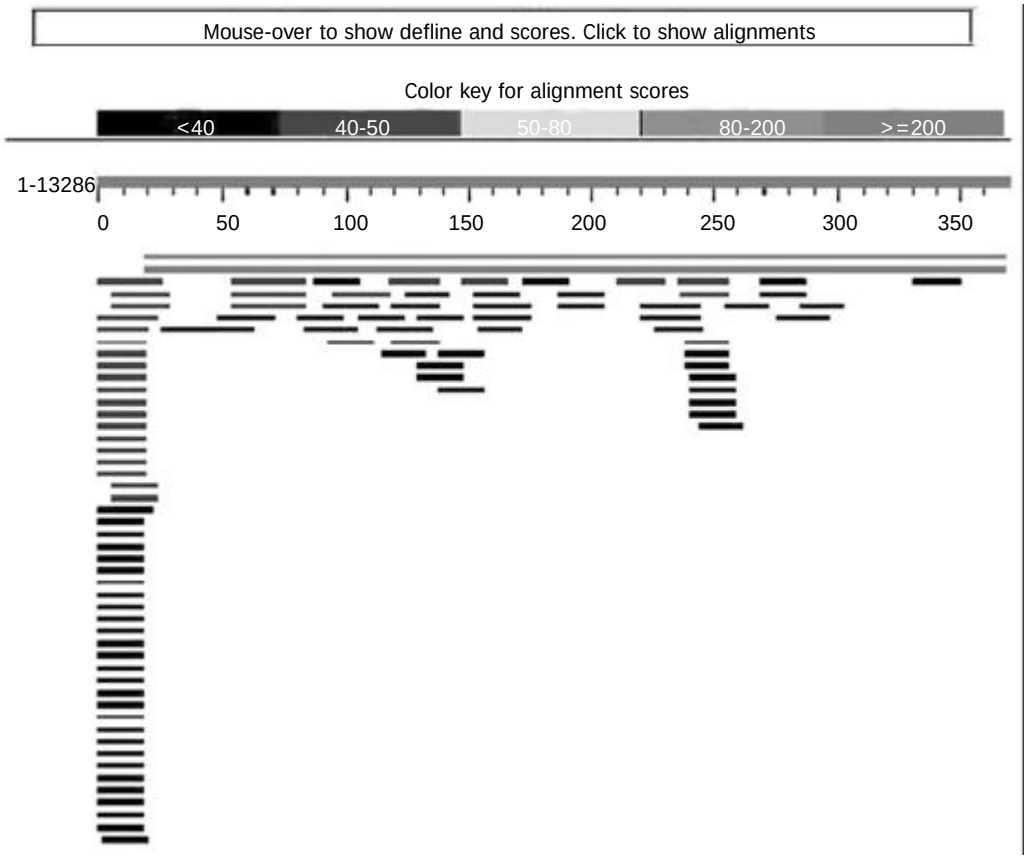
Score E

Sequences producing significant alignments: (bits) Value

gi|13937856|gb|BC007034.1|BC007034 Homo sapiens, metallothi... 755 0.0 **L U**
gi|37120|emb|V00594.1|HSTHIO Human mRNA for metallothionein... 698 0.0 **U**
gi|18043898|gb|BC019382.1| Mus musculus, metallothionein 2A... 682 0.0 **U**
gi|18370038|gb|AC074378.4| Homo sapiens chromosome 4 clone ... 613 e-172
gi|20270509|ref|NG_001158.1| Homo sapiens metallothionein 2... 605 e-170 **L**
gi|187530|gb|J00272.1|HUMMET2PS Human metallothionein-II ps... 605 e-170 **L**
gi|1495465|emb|X97260.1|HSMTISO2 H.sapiens mRNA for metallo... 597 e-168 **U**
gi|187529|gb|M13074.1|HUMMET2PG Human metallothionein II pr... 589 e-165
gi|38121|emb|V01532.1|MOTHI2 Monkey complementary DNA codin... 496 e-137
gi|20146733|gb|AC026461.9| Homo sapiens chromosome 16 clone... 450 e-123
gi|467308|gb|J00271.1|HUMMET2 Human metallothionein-II gene... 442 e-121 **L**
gi|187527|gb|M26637.1|HUMMET2AB Human metallothionein II mR... 389 e-105 **L**
gi|5174763|ref|NM_005953.1| Homo sapiens metallothionein 2A... 369 3e-99 **L U**

Figure 3 Blast results of MT2A.

GSS, or phase 0, 1 or 2 HTGS sequences)
Taxonomy reports
Distribution of 114 Blast Hits on the Query Sequence



Sequences producing significant alignments:		Score (bits)	E Value
gi|23334560|gb|AF422192.1|	Homo sapiens esophagus cancer-re...	696	0.0
gi|15282087|emb|AL117190.6|CNS01DRF	Human chromosome 14 DNA...	696	0.0

Figure 4 Blast results of AF422192.

Bioinformatics

Blast online (http://www.ncbi.nlm.nih.gov/blast) showed the genes identified by the yeast two-hybrid approach were metallothionein 2A(MT2A), metallothionein 1H, metallothionein 1G, ferritin, erythrocyte membrane protein band 4.2, hemoglobin, mitochondrial ribosomal protein S12, hypothetical protein FLJ10101, and a novel gene whose cDNA was found to have no strong homology to any other previously characterized gene whose GenBank accession No.is AF422192.

DISCUSSION

Protein-protein interactions played important roles in almost all events that took place in a cell[6]. Because proteins often assembled into large complexes to perform discrete activities, the characterization of the interaction pattern of a protein could provide considerable assistance in the elucidation of the functions of that protein[7]. The availability of complete genome sequences now permits the development of tools for functional biology on a proteomic scale. Among those, the yeast two-hybrid system is the choice to detect protein-protein interactions[8,9]. The ECRG2 gene is a novel candidate of tumor suppressor gene identified from human esophageal carcinoma. Identifying the function of ECRG2 gene product may provide opportunities to elucidate the esophageal cancer mechanisms and its role in tumor development and progression. The yeast-two-hybrid approach could find novel partners for known function proteins and identify the function of a novel protein by identifying well-characterized interacting partners[10]. So, we searched for associated proteins with a yeast-two-hybrid system using the ECRG2 cDNA fragment as baits.

Our results showed that ECRG2 gene did not activate transcription by itself and was suitable for yeast two-hybrid. On screening a human fetal liver cDNA library, we identified 9 putative clones as associated proteins, which included metallothionein 2A (MT2A), metallothionein 1H, metallothionein 1G. Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich, metal ion-binding proteins which are wildly distributed in various species. MTs were thought to be involved in heavy-metal detoxification, intracellular trace elements storage and scavenging free radicals. Recently, emerging data suggested that MTs had a close relationship with tumors. They might play important roles in carcinogenic and apoptotic process and differentiation of tumor cells[11-23]. In addition, MTs were attributed to affording tumor cell resistance to some important chemotherpeutic agents[24]. Using immunohistochemical-staining method, MTs have been localized intensively in various types of human tumors in organs and tissues such as skin, kidneys, prostate, tests, gallbladder, colon, breast and endometrium[25-34]. Since

human metallothioneins are closely linked with tumor, it is possible for us to understand the cellular functions of the ECRG2 protein through its linkage to MT2A. The other associated clone is a novel gene whose cDNA was found to have no strong homology to any other previously characterized gene whose GenBank Accession No.is AF422192. There are also 5 clones including ferritin, erythrocyte membrane protein band 4.2, hemoglobin, mitochondrial ribosomal protein S12 and hypothetical protein FLJ10101. Determination of a reliability score for each single protein-protein interaction, especially interaction of ECRG2 and MTs, permits the assignment of ECRG2 and unannotated proteins to biological pathways. A further understanding of the association between ECRG2 and MT should facilitate the functions of ECRG2 gene.

REFERENCES

1 **Lu SH**. Alterations of oncogenes and tumor suppressor genes in esophageal cancer in China. *Mutat Res* 2000; **467**: 343-353
2 **Zhou J,** Zhao LQ, Xiong MM, Wang XQ, Yang GR, Qiu ZL, Wu M, Liu ZH. Gene expression profiles at different stages of human esophageal squamous cell carcinoma. *World J Gastroenterol* 2003; **9**: 9-15
3 **Montesano R,** Hollstein M, Hainaut P. Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. *Int J Cancer* 1996; **69**: 225-235
4 **Xiong XD**, Xu LY, Shen ZY, Cai WJ, Luo JM, Han YL, Li EM. Identification of differentially expressed proteins between human esophageal immortalized and carcinomatous cell lines by two-dimensional electrophoresis and MALDI-TOF-mass spectrometry. *World J Gastroenterol* 2002; **8**: 777-781
5 **Su T**, Liu HL, Lu SH. Cloning and identification of cDNA fragments related to human esophageal cancer. *Zhonghua Zhongliu Zazhi* 1998; **20**: 254-257
6 **Blackstock WP**, Weir MP. Proteomics: quantitative and physical mapping of cellular proteins. *Trends Biotechnol* 1999; **17**: 121-127
7 **Emmert-Buck MR**, Gillespie JW, Pawletz CP, Ornstein DK, Basrur V, Appella E, Wang QH, Huang J, Hu N, Taylor P, Petricoin EE 3rd. An approach to proteomic analysis of human tumors. *Mol Carcinog* 2000; **27**: 158-165
8 **Fromont-Racine M**, Rain JC, Legrain P. Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. *Nature Genet* 1997; **16:** 277-282
9 **Toby GG**, Golemis EA. Using the yeast interaction trap and other two-hybrid-based approaches to study protein-protein interactions. *Methods* 2001; **24**: 201-217
10 **Chambers G**, Lawrie L, Cash P, Murray GI. Proteomics: a new approach to the study of disease. *J Pathol* 2000; **192**:280-288
11 **Zhang XH**, Takenaka I. Incidence of apoptosis and metallothionein expression in renal cell carcinoma. *Br J Urol* 1998; **81**: 9-13
12 **Tan Y**, Sinniah R, Bay BH, Singh G. Metallothionein expression and nuclear size in benign, borderline, and malignant serous ovarian tumours. *J Pathol* 1999; **189**: 60-65
13 **Takaba K**, Saeki K, Suzuki K, Wanibuchi H, Fukushima S. Significant overexpression of metallothionein and cyclin D1 and apoptosis in the early process of rat urinary bladder carcinogenesis induced by treatment with N-butyl-N-(4-hydroxybutyl) nitrosamine or sodium L-ascorbate. *Carcinogenesis* 2000; **21**: 691-700
14 **Jayasurya A**, Bay BH, Yap WM, Tan NG, Tan BK. Proliferative potential in nasopharyngeal carcinoma: correlations with metallothionein expression and tissue zinc levels. *Carcinogenesis* 2000; **21**: 1809-1812
15 **Hiura T**, Khalid H, Yamashita H, Tokunaga Y, Yasunaga A, Shibata S. Immunohistochemical analysis of metallothionein in astrocytic tumors in relation to tumor grade, proliferative potential, and survival. *Cancer* 1998; **83**: 2361-2369
16 **Hishikawa Y**, Kohno H, Ueda S, Kimoto T, Dhar DK, Kubota H, Tachibana M, Koji T, Nagasue N. Expression of metallothionein in colorectal cancers and synchronous liver metastases. *Oncology* 2001; **61**: 162-167
17 **Abdel-Mageed AB**, Agrawal KC. Activation of nuclear factor kappaB: potential role in metallothionein-mediated mitogenic response. *Cancer Res* 1998; **58**: 2335-2338
18 **Aloia TA**, Harpole DH Jr, Reed CE, Allegra C, Moore MB, Herndon JE D' Amico TA. Tumor marker expression is predictive of survival in patients with esophageal cancer. *Ann Thorac Surg* 2001; **72**: 859-866
19 **Joseph MG**, Banerjee D, Kocha W, Feld R, Stitt LW, Cherian MG. Metallothionein expression in patients with small cell carcinoma of the lung: correlation with other molecular markers and clinical outcome. *Cancer* 2001; **92**: 836-842
20 **Ebert MP**, Gunther T, Hoffmann J, Yu J, Miehlke S, Schulz HU, Roessner A, Korc M, Malfertheiner P. Expression of metallothionein II in intestinal metaplasia, dysplasia, and gastric cancer. *Cancer Res* 2000; **60**: 1995-2001
21 **Jin R**, Chow VT, Tan PH, Dheen ST, Duan W, Bay BH. Metallothionein 2A expression is associated with cell proliferation in breast cancer. *Carcinogenesis* 2002; **23**: 81-86
22 **Jayasurya A**, Bay BH, Yap WM, Tan NG. Correlation of metallothionein expression with apoptosis in nasopharyngeal carcinoma. *Br J Cancer* 2000; **82**: 1198-1203
23 **Cherian MG**, Howell SB, Imura N, Klaassen CD, Koropatnick J, Lazo JS, Waalkes MP. Role of metallothionein in carcinogenesis. *Toxicol Appl Pharmacol* 1994; **126**: 1-5
24 **Jasani B**, Schmid KW. Significance of metallothionein overexpression in human tumours. *Histopathology* 1997; **31**: 211-214
25 **Huang GW**, Yang LY. Metallothionein expression in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 650-653
26 **Rossen K**, Haerslev T, Hou-Jensen K, Jacobsen GK. Metallothionein expression in basaloid proliferations overlying dermatofibromas and in basal cell carcinomas. *Br J Dermatol* 1997; **136**: 30-34
27 **Zhang XH**, Jin L, Sakamoto H, Takenaka I. Immunohistochemical localization of metallothionein in human prostate cancer. *J Urol* 1996; **156**: 1679-1681
28 **Giuffre G**, Barresi G, Sturniolo GC, Sarnelli R, D'Inca R, Tuccari G. Immunohistochemical expression of metallothionein in normal human colorectal mucosa, in adenomas and in adenocarcinomas and their associated metastases. *Histopathology* 1996; **29**: 347-354
29 **Kuo T**, Lo SK. Immunohistochemical metallothionein expression in thymoma: correlation with histological types and cellular origin. *Histopathology* 1997; **30**: 243-248
30 **Shukla VK**, Aryya NC, Pitale A, Pandey M, Dixit VK, Reddy CD, Gautam A. Metallothionein expression in carcinoma of the gallbladder. *Histopathology* 1998; **33**: 154-157
31 **Zelger B**, Hittmair A, Schir M, Ofner C, Ofner D, Fritsch PO, Bocker W, Jasani B, Schmid KW. Immunohistochemically demonstrated metallothionein expression in malignant melanoma. *Histopathology* 1993; **23**: 257-264
32 **Goulding H**, Jasani B, Pereira H, Reid A, Galea M, Bell JA, Elston CW, Robertson JF, Blamey RW, Nicholson RA. Metallothionein expression in human breast cancer. *Br J Cancer* 1995; **72**: 968-972
33 **Douglas-Jones AG**, Schmid KW, Bier B, Horgan K, Lyons K, Dallimore ND, Moneypenny IJ, Jasani B. Metallothionein expression in duct carcinoma in situ of the breast. *Hum Pathol* 1995; **26**: 217-222
34 **Uozaki H**, Horiuchi H, Ishida T, Iijima T, Imamura T, Machinami R. Overexpression of resistance-related proteins (metallothioneins, glutathione-S-transferase pi, heat shock protein 27, and lung resistance-related protein) in osteosarcoma. Relationship with poor prognosis. *Cancer* 1997; **79**: 2336-2344

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