DOI:10.13471/j.cnki.j.sun.yat-sen.univ(med.sci).2004.0137

cDNA 微阵列和组织微阵列对卵巢上皮性肿瘤基因的表达

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摘　要: 【目的】结合 cDNA 微阵列和 RNA 与冰冻组织微阵列原位杂交的方法以寻找新的特异性卵巢癌候选癌基因,为卵巢癌的早期诊断和治疗提供理论依据。【方法】利用 cDNA 微阵列筛选在所有 3 种上皮性卵巢肿瘤中(卵巢浆液性交界性肿瘤、卵巢浆液性腺癌和卵巢子宫内膜样腺癌)显示有意义表达的基因,由 RNA 与冰冻组织微阵列原位杂交证实其结果。【结果】28 个基因在 > 70% 卵巢肿瘤中显示出高表达,18 个基因在 > 70% 卵巢肿瘤中低表达;干扰素诱导的转膜蛋白 1 (IITP1)被进一步用于 RNA 与冰冻组织微阵列原位杂交研究,其结果与 cDNA 微阵列研究结果相符合。【结论】将 cDNA 微阵列和 RNA 与冰冻组织微阵列原位杂交相结合是寻找卵巢癌候选癌基因的可行方法,研究中显示有意义表达的基因有可能作为新的上皮性卵巢癌候选癌基因。

关键词:上皮性卵巢癌;cDNA 微阵列;组织微阵列;癌基因
中图分类号:R737. 31; R730. 43　　文献标识码:A　　文章编号:1672-3554(2004)05-0399-05

Gene Expression Profiles to Epithelial Ovarian Tumors by Using cDNA Microarray and Tissue Microarrays

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Abstract: 【Objective】Combining cDNA microarray with RNA in situ hybridization on frozen tissue microarray to identify novel candidate oncogenes and provide possible theoretical basis for early diagnosis and treatment of ovarian cancer. 【Methods】cDNA microarrays were used to seek significantly expressed genes in 3 types of ovarian tumors (serous borderline ovarian tumors, serous ovarian cancers, and endometrioid ovarian carcinomas). RNA in situ hybridization on frozen tissue microarray was used to confirm the finding from cDNA microarrays. 【Results】In the study of cDNA microarray, 28 genes were over-expressed and 18 genes were under-expressed in > 70% ovarian tumors. Interferon induced transmembrane protein 1(IITP1) was further validated by RNA in situ hybridization on frozen tissue microarray. 【Conclusion】The methods through combining cDNA microarray with RNA in situ hybridization on frozen tissue microarray, is an ideal choice for identifying novel oncogenes. The genes identified in this study might be the new candidate oncogenes of ovarian cancer.

Key words: epithelial ovarian tumors; cDNA microarray; tissue microarray; oncogene
[J SUN Yat-sen Univ (Med Sci), 2004, 25(5): 399 ~ 403]

卵巢癌居妇科肿瘤死亡率之首,主要由于早期缺乏症状和有效的诊断方法,其发病的分子机制尚不清楚。已发现 HER-2/neu、CA125 等癌基因及 p53、BRCA1 等抑癌基因与卵巢癌有关[1-3]。但

收稿日期: 2004-02-05
基金项目: 瑞士国家自然科学基金资助项目(National Scientific Fund of Switzerland No. E430573)
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卵巢癌的发生和发展是一个多基因参与、多阶段发展的过程,仅仅研究一个或几个肿瘤相关基因的结构和功能,不能全面揭示细胞癌变的分子机制,cDNA微阵列为全面系统分析卵巢癌癌基因提供了新的手段。本研究中,我们首先运用cDNA微阵列筛选在上皮性卵巢肿瘤有意义表达的基因,同时与膀胱癌及其它一些组织基因表达进行比较,然后随意选出有代表意义的基因采用RNA与冰冻组织微阵列原位杂交技术进一步进行验证。我们试验的目的是试图找到与可用于早期诊断卵巢癌的特异性肿瘤标志物相关的候选癌基因,为卵巢癌的早期诊断和治疗提供可靠的理论依据。

1 材料和方法

1.1 cDNA微阵列试验

1.1.1 材 料 ①试验组来自卵巢、输卵管切除术和卵巢癌细胞减灭术的快速冰冻组织:3例卵巢浆液性交界性肿瘤、7例卵巢浆液性腺癌、5例卵巢子宫内膜样腺癌;② 14种膀胱癌细胞株;③ 5例正常肾脏组织; ④对照组选用人类共同参考RNA(UHRR, STRATAGENE, Stanford University, USA),它含有10种来自人类肿瘤细胞株的优质RNA。

1.1.2 cDNA微阵列 产自美国Ambion公司(详见Ambion公司网站www.ambion.com),每1张芯片含有16 800个插入cDNA(或8 400对经PCR扩增的cDNA)和27个对照物,含有7 236对已知基因和1 159对ESTs,其中有25对与凋亡相关、48对与interleukin相关和88对癌基因。

1.1.3 提取mRNA 用Trizol试剂(1 mL Trizol试剂/ 100 mg组织 GIBCO, Basel, Switzerland)从0.05~0.1 g冰冻组织粗提取总RNA,无水乙醇沉淀加以纯化,用紫外线分光光度仪测定浓度。通过琼脂糖凝胶电泳测定并用溴化乙锭评估总RNA的完整性。再使用DynaBead试剂盒(Dynal, Oslo, Norway)从总RNA提取mRNA。

1.1.4 探针合成和标记 用250~500 ng mRNA逆转录合成cDNA(Ambion UltraArray试剂盒,Ambion Inc, Austin, USA),同时予(α-^{33}P) dATP ($7.4\sim14.8\times10^{10}$ kBq/mmol Amersham Pharmacia Ltd, UK)加以标记,去核苷酸旋转柱(NucAway Spin Column,Ambion)除掉未被利用的核苷酸片段。

1.1.5 杂 交 标记的cDNA与cDNA微阵列混合,在充足杂交液里、60 ℃温度下杂交14~16 h,2×SSC(含5 g/L SDS)和0.5×SSC(含5 g/L SDS)在60 ℃分别清洗30 min。杂交后的cDNA微阵列置于分辨率高的磷光图像荧光屏上(Canberra Packard, Zürich, Switzerland)曝光4 h,再通过旋转磷光图像仪成像(Packard, Tokio, Japan)。

1.1.6 结果分析 杂交后的图像输入ArrayVision (Imaging research inc, Ontario, Canada)软件包, 计算每一微阵列点的原始杂交强度值,与所有微阵列点平均强度值相比将其规格化。各实验组规格化杂交强度值/对照组规格化杂交强度值(normalized ratio),再将卵巢肿瘤该比值与膀胱癌和正常肾脏组织比较(mean ratio),相差 > 1.2倍为高表达的基因;而 < 0.5(= -1.2)为低表达的基因;介于两者之间的为没有表达差异的基因。筛选出的基因必须同时满足另一成对基因也显示相同表达的条件。

1.2 RNA与冰冻组织微阵列原位杂交试验

1.2.1 卵巢肿瘤冰冻组织微阵列 由39例卵巢肿瘤组织组成的冰冻组织微阵列购自Diomeda Life Sciences公司(Basel, Switzerland),含26例卵巢浆液性腺癌、9例卵巢子宫内膜样腺癌、4例卵巢浆液性交界性肿瘤,经卵巢、输卵管切除术和卵巢癌细胞减灭术并经快速冰冻取得。用微阵列制作仪取得组织小圆柱块(直径0.6 mm,高3~4 mm),直接放入充满Tissue-Tek标准(大小类似石蜡收受模块)微阵列冰冻收受模块中,每2个微阵列块相距1 mm,制作中模块四周用干冰以防融化;用冰冻切片机和玻片制成4~10 μm组织微阵列切片[4,5]。

1.2.2 寡核苷酸的设计和标记 Vector NTI软件包设计IITP1的寡核苷酸(详见www.vectornti.co.kr)。分别用^{33}P标记每个寡核苷酸,再合为一体以达到最佳标记强度,用QIAGEN去除核苷酸试剂盒kit (QIAGEN, Basel, Switzerland)清洗未结合的核苷酸。

1.2.3 杂 交 标记后的探针与25 μL Cot1 DNA, 15 μL 5 mol/L DTT和485 μL杂交液在42 ℃混合1 h后,冰冻组织微阵列与标记的探针及其杂交液一起在42 ℃杂交约36 h,用1×SSC在55 ℃清洗4次,每次15 min后,置于1×SSC溶液室温下1 h,蒸馏水清洗30 s,600 mL/L酒精脱水30 s,950 mL/L酒精脱水30 s,空气干燥10 min。放射

显像可曝光 48 h 到高分辨率磷光图像片上，用旋转磷光图像仪捕捉成像。

1.2.4　**结果分析**　图像被输入 ArrayVision 软件包，计算每一芯片点纠正背景后的放射强度值，比较代表不同卵巢肿瘤组织的各微阵列点放射强度值。

1.2.5　**涂乳胶显像**　在暗室内，将干燥的组织微阵列玻片浸入乳胶中 5 s，空气中干约 5 min，继续留在室温下 1 h 直到玻片全干，然后将玻片储存在 2～8 ℃2～4 周。将玻片取出置室温下 30～60 min，显影液涂于玻片 5 min，停止反应液中止反应，固定液固定 10 min。玻片放入 Harris 溶液 4 min，蒸馏水清洗，放入 HCl 液中 5 s，蒸馏水清洗，乙醇脱水，二甲苯脱蜡，盖玻片覆盖组织微阵列玻片。

2　结　果

2.1　cDNA 微阵列研究筛选有意义表达的基因

与对照组相比，有 28 个基因显示在所有卵巢肿瘤高表达，18 个基因显示在所有卵巢肿瘤低表达 表 1,2)。

表 1　卵巢肿瘤高表达的基因

Table 1　Frequently over-expressed genes in ovarian tumors

Gene	Unigene	Mean ratio	Expression(%)	Function
Putative selenocysteine lyase	Hs. 285306	9.2	100	May decompose L-selenocysteine to L-alanine and elemental selenium, may function with selenophosphate synthetase in selenoprotein synthesis
Interferon induced transmembrane protein 1(IITP1, LEU13)	Hs. 146360	5.9	80	Interferon-inducible transmembrane protein 1; involved in relaying antiproliferative and ho motypic adhesion signals
Gap junction protein (CX43)	Hs. 74471	5.9	93	Connexin 43, a cardiac gap junction protein; may act in synchronizing heart contraction and embryonic development
RAD1 (S. pombe) homolog	Hs. 7179	5.2	86	3′ to 5′ exonuclease that has predicted roles in DNA damage-activated mitotic and meiotic cell cycle checkpoints
KIAA0483 protein	Hs. 64691	4.8	93	
PRS	Hs. 2910	3.9	93	Phosphoribosyl pyrophosphate synthetase 2; generates the PRPP needed for initiation of purine biosynthesis
Protein phosphatase 3 (CNA2))	Hs. 151531	3.5	93	Catalytic subunit of calmodulin regulated protein phosphatase 3; regulates activity of tran scription factors involved in signal transduction and growth control
Fe-S protein 8 (TYKY)	Hs. 90443	3.1	80	Fe-S subunit of NADH-ubiquinone oxidoreductase (complex I); transports electrons from NADH to ubiquinone
PPLA2	Hs. 992	3	86	Group IB pancreatic phospholipase a2; hydrolyzes the phospholipid sn-2 ester bond;
Low density lipoprotein-related protein-associated protein1 MRAP	Hs. 75140	2.9	80	α-2-macroglobulin receptor-associated protein; prevents premature binding of ligands to receptors; enables correct folding and export from the ER of α-2-macroglobulin receptor, affects interactions between plasma membranes and basement membranes
Guanylate cyclase 2D, membrane	Hs. 1974	2.7	100	Retinal guanylate cyclase; membrane bound enzyme that converts GTP to cGMP
Hypothetical protein FLJ10461	Hs. 122579	2.6	93	Strongly similar to a region of murine Ect2
Period (Drosophila) homolog 2	Hs. 153405	2.6	80	Period homolog 2; putative circadian clock protein; has a PAS dimerization domain
Membrane-spanning 4-domains, subfamily A, member 1(BP35)	Hs. 30	2.5	73	Beta subunit of the high affinity IgE receptor; plays an important role in allergic reaction
H3 histone family, K(H2FK)	Hs. 70937	2.3	93	Member K of the H3 histone family; involved in compaction of DNA into nucleosomes
Immunoglobulin family, member 6	Hs. 135194	2.3	73	Member of the CD8 family of receptors; may act in antigen uptake or homing/recirculation of dendritic cells; may contain tyrosine-phosphorylation sites
Clone NIBB11	Hs. 7057	2.2	80	Homo sapiens Cri-du-chat region mRNA, clone NIBB11
Nucleoporin-like protein 1(NLP-1)	Hs. 168352	2.2	86	Similar to nucleoporins; interacts with proteins involved in nuclear export
Ovarian carcinoma antigen CA125	Hs. 277721	2.2	73	Contains a B-box/coiled coil motif。chromosome 17, surface marker 2
TIMP1	Hs. 5831	2.1	80	Inhibits collagenase IV (MMP2) and stimulates growth of erythroid cells
Myosin IE	Hs. 121555	2.1	80	Highly similar to class I myosin; may bind proline-rich peptides
CCAAT-box-binding transcription	Hs. 184760	2	86	Binds to a CCAAT-box element, contributes to gene-specific transcriptional activation
E2F transcription factor 5,	Hs. 26776	1.9	86	Neurotrophic tyrosine kinase receptor, type 3; binds neurotrophin-3 (NTF3)
TRKC	Hs. 2331	1.8	73	E2F family transcription factor 5, p130-binding; involved in cell cycle regulation
DKFZP566H073 protein	Hs. 7158	1.8	86	May mediate protein-protein interactions; contains a C3HC4 type (RING) zinc finger
KIAA0556 protein	Hs. 30512	1.7	93	
Regulator G-protein signalling12	Hs. 78281	1.5	80	Negatively regulates G protein-coupled receptor signalling
CACH1	Hs. 1294	1.5	73	Dihydropyridine receptor; involved in coupling of excitation and contraction in muscle

表 2 卵巢肿瘤低表达的基因

Table 2 Frequently under-expressed genes in ovarian tumors

Gene	Unigene	Mean ratio	Expression (%)	Function
Hemoglobin, gamma A(HBGA)	Hs. 283106	−4. 8	100	
Trefoil factor 1 (BCEI)	Hs. 1406	−4. 7	100	Maintains the mucosal surface barrier and stimulates repair processes
Farnesyl diphosphate synthase (FPS)	Hs. 77393	−3. 8	93	Farnesyl pyrophosphate synthetase; part of the cholesterol synthesis pathway
Ribosomal protein L37	Hs. 179779	−2. 9	100	Ribosomal protein L37; component of the large 60S ribosomal subunit
Ribosomal protein L38	Hs. 2017	−3. 0	80	Ribosomal protein L38; component of the large 60S ribosomal subunit
Ribosomal protein S10	Hs. 76230	−2. 9	86	Ribosomal protein S10; component of the small 40S ribosomal subunit
Ribosomal protein S24	Hs. 180450	−2. 2	80	Ribosomal protein S24; component of the small 40S ribosomal subunit
Immunoglobulin lambda locus	Hs. 181125	−2. 4	80	
Immunoglobulin lambda joining 3	Hs. 289110	−1. 9	73	
Immunoglobulinlambda polypeptide3	Hs. 251397	−1. 7	80	Similar to beta − glucuronidases
Lectin, galactoside − binding, soluble, 1	Hs. 227751	−2. 6	100	beta − galactoside − binding lectin, acts as a cell growth regulatory factor
Ubiquinol − cytochrome c reductase hinge protein	Hs. 73818	−2. 5	93	Ubiquinol − cytochrome c reductase hinge protein; hinges cytochrome c With cytochrome c1
Peroxisomal biogenesis factor 11A	Hs. 31034	−2. 0	93	May recruit ADP − ribosylation factor, coatomers; highly similar to rat
GDP − mannose pyrophosphorylase B	Hs. 28077	−1. 8		Similar to S. cerevisiae Psa1p; as a mannose − 1 − phosphate guanyltransferase
			80	Precursor of oxytocin and its carrier protein neurophysin I
Pyruvate kinase, muscle	Hs. 198281	−1. 7	86	
Oxytocin, prepro − neurophysin I	Hs. 113216	−1. 6	80	Drecursor of xxytocin and its carrier protein neurophysin 1
Testis enhanced gene transcript	Hs. 74637	−1. 6	73	Contains seven predicted transmembrane domains
Neuroendocrine secretory protein 55	Hs. 113368	−1. 6	80	

Mean ratio: normalized ratio of ovarian tumors. / other tissue types; %: the patients' percentage of Over-expression or under-expression; over-expressed genes: normalized ratio > 1. 2; Under-expressed genes: normalized ratio < −1. 2; Hs., unigene cluster IDs

2. 2 RNA 与冰冻组织微阵列原位杂交试验

cDNA 微阵列结果发现 IITP1 属于在 3 种卵巢肿瘤组织均显示高表达,而在所有膀胱癌细胞株和正常肾脏组织不显示表达的基因,故进入 RNA 与冰冻组织微阵列原位杂交试验研究,结果显示与 cDNA 微阵列试验结果相符合(图 1)。组织微阵列上 36/39 例(92%)的上皮性卵巢肿瘤显示有 IITP1 基因表达,其中 25/26(96%)的卵巢浆液性腺癌显示有 IITP1 基因表达,9/9 例(100%)卵巢子宫内膜样腺癌显示有 IITP1 基因表达,而 2/4 例(50%)卵巢浆液性交界性肿瘤有 IITP1 基因表达。

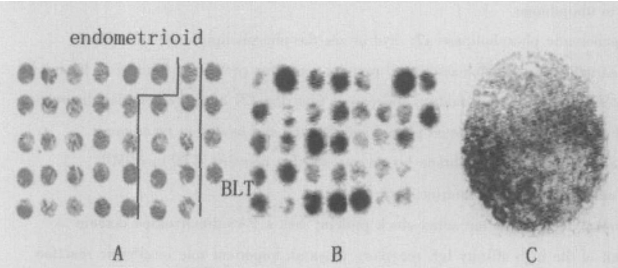

图 1 RNA 与卵巢肿瘤冰冻组织微阵列原位杂交试验显示 IITP1 基因表达情况

Fig. 1 Gene expression of IITP1 from RISH on frozen ovarian tumor array

A. Overview of frozen epithelial ovarian tumor microarray with 39 tissue cylinders and locations of different ovarian tumor subtypes (H&E, magnification, ×4. 2) Endometrioid: ovarian endometrioid carcinomas; serous: ovarian serous carcinomas; BLT: ovarian serous borderline tumors

B. Original image of differential expression of IITP1 on ovarian tumor microarray (original magnification, ×4. 2) Empty spots: no gene expression; Grey-black: gene under-expression to over-expression

C. Hypercoat emulsion image of differential expression of IITP1 on ovarian serous carcinoma (labled with 33P, original magnification, ×84)

3 讨 论

研究发现:与对照组和膀胱癌细胞株及正常肾脏组织相比,28 个基因在大多数卵巢肿瘤高表达,18 个基因在大多数卵巢肿瘤低表达,IITP1、SCL、CX43、RAD1 属于在大多数卵巢肿瘤有较高表达水平的基因,提示这些基因有可能成为卵巢肿瘤所特有的候选癌基因,经 RNA 与冰冻组织微阵列原位杂交进一步证实的 IITP1 属于 ifn 诱导的转膜蛋白家族,联系到信号转导的细胞表面受体,是一多元复杂的混合物包括在抗增值和黏附信号转导过程中,动物试验结果支持 IITP1 具有可能抗肿瘤作用,但人类临床试验显示这种效果仅限于在少数几种癌瘤中,它的制癌机理仍然不清楚[6]。本研究首次报道 IITP1 可能作为有别于其它肿瘤的卵巢癌所独有的致癌基因,其作用机理仍值得进一步探

讨。SCL 是硒代胱氨酸裂解酶，在硒蛋白合成过程中与硒磷酸合成酶共同起作用，它在肿瘤恶性转化过程中所起作用尚不明了[7]。CX43 在控制细胞生长方面发挥重要作用，几种研究证实 CX43 在大多数肿瘤细胞表达降低；与卵巢浆液性腺癌相比，正常卵巢上皮细胞显示较高水平 CX43 表达，在我们的研究中，CX43 在卵巢肿瘤表达较参照组高 5 ~ 8 倍[8]。RAD1 是一与细胞修复有关的蛋白，在细胞响应 DNA 的损害方面发挥很重要的作用[9]，在我们的研究中，RAD1 显示在卵巢肿瘤表达较参照组高 5 倍。另一方面，与对照组和膀胱癌细胞株及正常肾脏组织相比，HBGA、BCEI、FPS 显示在卵巢肿瘤表达相对水平较低，说明这些基因属于与其它种类恶性肿瘤有关的癌基因，而非与卵巢癌有关的癌基因。BCEI 在很多肿瘤如乳腺癌、胰腺癌、胃癌、小肠癌中表达，也在正常胃黏膜表达[10]；FPS 在胆固醇合成中起作用，据报道膀胱癌较膀胱良性肿瘤显示较高表达[11]。它们的制癌机理尚不明了。特别值得一提的是：目前临床上用于诊断和监测卵巢上皮细胞癌治疗效果的有效指标 CA125 和已知的在卵巢上皮细胞癌显示较高表达的 TIMP-1[12]，也均在本研究中得以进一步证实。在本研究中发现的这些基因可能是卵巢上皮细胞癌特异性的候选癌基因，值得进一步探讨。

欲获取 cDNA 微阵列试验可靠而精确的基因表达信息，主要依赖于试验组和对照组标本间杂交有效性的可比度。正常卵巢上皮细胞通常被考虑作为卵巢癌基因表达研究的参照物，但其来源有限，故极难获取。本研究选用了由 10 种人类肿瘤细胞株组成的高质量的总 RNA 混合物 (UHRR) 作为对照物，不光为不同的 cDNA 微阵列试验基因表达资料的精确和重复比较性提供了一种共性，同时使基因表达在不同实验室的 cDNA 微阵列试验也具有可比性。更重要的是，使卵巢肿瘤在 1 次试验中有与更多的其它种类的肿瘤基因表达相比较的机会，为寻找有别于其它种类肿瘤的特异性的卵巢肿瘤候选癌靶基因提供了简捷方便的途径。

我们实验室新发展的 RNA 与冰冻组织微阵列原位杂交技术，具有一次性获取基因在成百上千种不同种类的组织内表达信息的优势[6-8]，较传统的 PCR 及 Northern blot 方法具有省时、省力和节省开支的无可比拟的优越性，我们的研究表明该技术用于验证来自 cDNA 微阵列试验所发现的癌基因的可信性，并进一步探索其与临床分期、肿瘤病理分级和组织学分类的关系，是一种完全可行的研究手段。

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编辑　黄小延）