Differential gene expression profile reveals overexpression of MAP3K8 in invasive endometrioid carcinoma

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Summary

Gene microarray technology is highly effective in screening for differential gene expression and has hence become a popular tool in the molecular investigation of cancer. In the present study, cDNA microarrays containing 2,000 different genes were used to analyze gene expression profiles in ten human postmenopausal endometrioid-paired carcinoma specimens versus corresponding adjacent normal tissue to identify differentially expressed genes. In our study several genes were found differentially expressed. One of them was the MAP3K8, a gene that has never been described to be overexpressed in this kind of malignancy. To validate the differential expression of this gene as well as the membrane array, we performed semiquantitative reverse transcription-PCR analysis. MAP3K8 was found overexpressed in 30% of the endometrial carcinoma samples. To the best of our knowledge this is the first report showing the MAP3K8 oncogene linked to human endometrial carcinoma suggesting that it may be another molecule involved in human endometrial cancer.

Key words: cDNA array; Endometrial carcinoma; MAP3K8.

Introduction

Endometrial cancer ranks among the most common gynecologic malignancies in the USA, alongside ovarian cancer [1]. In Brasil it is estimated that at least 11% of the gynecologic malignancies arise in the endometrium.

In the majority of cases, approximately 75%, the tumor is confined to the uterus at the time of diagnosis and has a relatively good prognosis [2]. In general, tumor development and tumor progression are thought to be driven by genetic alterations.

Therefore, insight into the molecular mechanisms involved in the progression of endometrial carcinoma is important when searching for new tools to improve the outcome of patients with advanced/recurrent endometrial carcinoma.

With the development of cDNA array technology, a method which has become available to study the expression levels of a large range of genes in one hybridization, requiring only a small amount of RNA. Using this technique, a gene expression profile of a single tissue sample can be made.

Some recent studies have reported differential gene expression between normal and neoplastic endometrium and between histological types of endometrial carcinoma [3, 4]. The aim of this study was to determine gene expression profiles in ten human endometrial-paired carcinoma specimens versus corresponding adjacent normal tissue by cDNA array to identify differentially expressed genes.

Revised manuscript accepted for publication May 8, 2006

Materials and Methods

Tissue samples

Ten pairs of normal and endometrial tumor samples (endometrioid carcinomas) that had been previously pathologically characterized were used. Samples were collected from 2002 to 2004; all patients were in the postmenopausal period with age ranging from 55 to 70 years old (mean 60). These tumors were diagnosed in the Pathology Department of the Federal University of São Paulo, Brazil. Sections of liquid nitrogen frozen samples were split for confirmatory histology by H&E staining and RNA isolation. Only tumor samples containing at least 70% of malignant tissue were included. All tumors were classified as endometrioid carcinomas.

RNA extraction, probe synthesis, and hybridization on cDNA arrays

Total RNA was isolated using TRIZOL reagent (Invitrogen, São Paulo) as indicated by the manufacturer. Purity of isolated RNA was evaluated spectrophotometrically by the A260/A280 absorbance ratio. The total RNA quality was checked by formaldehyde/agarose gel electrophoresis. Prior to reverse transcriptions, two pools of RNA were obtained by mixing 2 µg of total RNA from each individual case in two tubes representing group I (normal tissue) and II (tumor tissue). In order to obtain radiolabeled cDNA probes, the two RNA pools, containing 50 µg RNA each, were reverse transcribed in the presence of oligodT primers and α-[33P] dATP (2000 Ci/mmol) by using the Superscript II kit (Invitrogen) following the manufacturer's instructions. Probes were purified by Probe Quant G50 micro-columns (Amersham Pharmacia Biotech). To analyze different gene expression between group I and II, four human cDNA expression-array membranes containing 2,000 genes were used (two times). Membranes were assembled at the microarray facility – "Universidade Estadual Paulista" – by using the ORESTES clones obtained from the "Fundação de Amparo a Pesquisa do Estado de São Paulo"/Ludwig Institute for Cancer

Table 1. — *Clinical and histopathological characterization of the patients.*

	Tumor histology	Stage	Grade of Differentiation	Myometrial invasion	Lymph/Vascular invasion	Lymph node metastasis	Over expression	Follow-up
1	Endometrioid	IIB	G2	< 50%	0	0	YES	Death
2	Endometrioid	IC	G1	> 50%	0	0	YES	Death
3	Endometrioid	IC	G1	> 50%	0	0	YES	Followed
4	Endometrioid	IIB	G3	< 50%	0	0	NO	Followed
5	Endometrioid	IB	G1	< 50%	0	0	NO	Followed
6	Endometrioid	IIIA	G3	< 50%	0	0	NO	Followed
7	Endometrioid	IIIB	G3	> 50%	1	0	NO	Followed
8	Endometrioid	IC	G2	> 50%	0	0	NO	Followed
9	Endometrioid	IC	G2	> 50%	1	0	NO	Followed
10	Endometrioid	IIIA	G3	> 50%	1	0	NO	Death

Research - Human Cancer Genome Project [5] and were sequence verified. Whenever possible, each gene was represented by two clones corresponding to different regions of the complete cDNA and each cDNA clone was printed in duplicate onto nylon membranes. Hybridization took place at 42°C in an overnight reaction containing 20 x standard saline citrate (SSC), 50 x Denhardt's solution, 50% formamide, 10 x sodium dodecyl sulphate (SDS) and 100 µg/ml salmon-sperm DNA. The filters were washed at 50°C in 1 x SSC/0.1% SDS for 15 minutes. Images were obtained by scanning the membrane in a storage phosphor system (Cyclone TM – Packard BioScience Company, Meriden, CT, USA). cDNA targets on the arrays were located using grid overlays and spot intensities were subsequently measured (ArrayVision software using gel files).

Data analysis

The expression ratios of the duplicated spots on the array were averaged. For statistical analysis, we selected genes with expressions that differed by a factor of at least 3-fold with respect to the reference pool in 30% of the patients. This selects genes with large variation in expression levels across the ten pairs of samples and ensures that the considered genes do show relevant differences with respect to the pool, at least doubling or halting of expression levels, so that these genes can be regarded as effectively repressed or overexpressed. By requiring that the repression or overexpression was shown by at least 30% of the patients, spurious results were avoided due to few outlying patients. To find a set of genes that were differentially expressed in the two groups, we used Welch's t test, which does not require equal variances between groups [6]. However, because we were testing for the differential expression of many genes, we needed to account for multiple testing to avoid an excessive number of false-positive results. Thus, we used the step-down maxT method [7, 8]. This method controls the family-wise error rate but is more powerful than traditional single-step procedures because it takes into account the order of the p values, makes successively smaller adjustments, and also considers covariance between genes. Because the sample size was small, the adjusted p values were obtained by random permutation using 50,000 random permutations. We considered genes to be differentially expressed in the two groups if their adjusted p value was 0.05. Statistical comparison was performed with the POMELO program (http://www.genoma.wi.mit.edu/MPR/software).

RT-PCR: Validation of array data by semiquantitative RT-PCR was performed by using equal amounts of total RNA from each individual patient (normal and tumor tissue); samples were then submitted to reverse transcription by using Superscript II RNAse H reverse transcriptase (Invitrogen) in a final volume of 20 µl according to the manufacturer's instructions. Primers were designed by the Prime3 program (http://www-genome.wi.mit.edu/cgibin/primer/primer3.cgi/primer3_www.cgi). The cytoplasmic beta actin (ACTB) gene was used to normalize the reactions. Two microliters of the reverse transcribed cDNA were amplified in a final volume of 100 µl by PCR under standard conditions: 1.5 mM MgCl2, 125 µM dNTP and 2.5 U Taq polymerase using specific primers at a 10 µM concentration. Primers and cycling parameters were: Cytoplasmic beta actin actin: 375 bp; 32 cycles (94°C,1 min/55°C,30 sec/72°C,30 sec) Sense: 5'- CGTGACATTAAGGAGAAGCTG -3'; Antisense: 3' CTCAGGAGGAGCAATGATCTTGA -5'. MAP3K8/Cot: 225 bp; 25 cycles (94°C,1 min/55°C,30 sec/72°C,30 sec) S: 5'- GTC CAT TTT GGG GAG TGA TG -3'; AS: 3'- GAG GAT TTG CTT GCT TTT GC -5'. The amplified PCR products were separated on 2% agarose gel containing 0.1 µg/ml ethidium bromide. The visualized bands were analyzed semi-quantitatively using imagescanning densitometry (Kodak EDAS 120) (Figure 1).

Results

Fifty differentially expressed genes were found in the present study (Table 1) when comparing normal atrophic endometrium with its tumoral counterpart. The MAP3K8 gene was found overexpressed in three out of ten tumor samples. Two out of the three patients showing overexpression of this gene died during the two years of follow-up and one of them showed more than 50% of tumor myometrial invasion. Regarding clinical stage the three patients were at Stages IIB, IC and IC and the differentiation grades were respectively G2, G1 and G1. None of them showed lymph/vascular invasion or lymph node impairment. Clinical and histopathological findings from all patients are displayed in Table 1.

Discussion

Ten pairs of endometrial carcinoma and their respective adjacent normal tissue were studied (Figure 1). Our main goal was to identify differences in gene expression in tumor tissues using the own normal counterparts.

All patients were in their postmenopausal period and therefore the results shown here represent differences in gene expression between atrophic endometrium and its tumoral counterpart.

Tumor samples were previously analyzed in order to include samples with at least 70% of tumor cells. A list

Figure 1. — Identification of genes differentially expressed in endometrium tumors. Schematic representation of our experimental design. (T, N) Representation of the dissecting procedure where only the invasive portion of an adenocarcinoma was excised and processed for RNA extraction. Each RNA sample was hybridized with two identical arrays. Representative image of our nylon array after hybridization with [α-^{33}P]dCTP-labeled cDNA. Signals were captured on a phosphorimager and data was acquired by the ArrayVision software using.gel files. Semi-quantitative RT-PCR was used to confirm differential expression in each individual samples.

Underexpression in tumor tissue

MMP13	-3,01	NM_02427
CFLAR	-3,04	NM_00387
GNG5	-3,06	NM_005274
GPRK6	-3,08	NM_001004106
TPT1	-3,10	NM_003295
KIR2DS2	-3,14	NM_012312
PEAA15	-3,15	NM_003768
P53R2	-3,19	AB166670
MDM4	-3,20	NM_002393
Neural adhesion molecule	-3,44	NM_181351
GNAS	-3,47	NM_080426
CTSD	-3,48	NM_001909
FN1	-3,62	NM_001909
ERC05	-3,67	NM_000123
NID	-3,88	NM_002508
TGFB	-3,92	NM_003597
TFAP4	-3,92	NM_003223
COL1A1	-4,00	NM_000088
TERA	-4,06	CK905319
H3F3A	-4,14	NM_002107
FGFintrac	-4,67	NM_198897
PRCC	-4,70	NM_199416
STRN	-4,78	NM_003162
PTK2	-4,83	NM_005607
HIRA	-5,03	NM_015700
E2F4	-5,73	NM_00195

USP11	8,62	NM_004651
HSMPP8	8,48	NM_017520
STIP1	6,08	NM_005861
CALM2P4	4,67	U44758
MAFF	4,29	NM_152878
DED	4,24	BD217303
MMP10	3,97	NM_002425
THRA	3,86	NM_199334
GRB2	3,84	NM_203506
SMAP	3,84	NM_014267
HSPC154 protein	3,79	NM_014177
pp2A	3,78	NM_181897
BTG2	3,69	NM_006763
T-STAR	3,62	AF069681
adaptor related prot. complex2	3,41	NM_178548
NUDT3	3,32	NM_006703
STAT2	3,23	NM_005419
ATP1B	3,22	NM_001001787
MAP3K8	3,18	NM_005204
TIMP3	3,17	NM_000362
WWP2	3,09	NM_199424
TP73	3,06	NM_003722
EGFR	3,06	NM_201283

Overexpression in tumor tissue

Figure 2. — Genes with differential expression in endometrioid tumors. Based on data presented the genes are listed (with respective folding differences indicated within brackets) with more than 3-fold differences. In dark gray, genes with lower expression in tumor tissue values; in light gray, genes with higher expression in tumor tissue.

containing all differentially expressed genes are shown in Figure 2. In Table 1 clinical and histopathological aspects of all patients as well as expression of MAP3K8 are shown. In order to validate differential gene expression we choose the MAP3K8 (Cot or Tpl-2) due to the fact that we were interested in the investigation of non-previously reported genes to be overexpressed in endometrial cancer. Validation was performed individually in all samples (Figure 1) making the identification of the overexpression of this gene possible in 30% of our tumor samples.

The MAP3K8 gene was originally identified in human thyroid tumor DNA as a gene capable of transforming SHOK (Syrian Hamster Osaka Kanazawa) cells [9]. By analysis of somatic cell hybrids and by fluorescence in situ hybridization, the MAP3K8 gene was mapped to chromosome 10p11.2 [10].

This gene was identified as a transforming gene from a human lung adenocarcinoma and has one 3-prime end mutation characterized in its cDNA. The mutation was localized to MAP3K8 exon 8 and confirmed in the primary tumor DNA.

Both wildtype and mutant MAP3K8 cDNAs transformed NIH3T3 cells, but the transforming activity of the mutant was much greater than that of the wildtype. That was the first report of a mutation in the MAP3K8 gene occurring in a primary tumor, but the authors concluded that mutational activation of the gene is a very rare event [11].

Present evidence has demonstrated that MAP3K8 is part of the pathways that significantly stimulate mitogen-activated protein kinases (MAPK). MAPK activity, in its turn, is necessary for the stimulation of the c-jun proto-oncogen promoter region during MAP3K8-induced neoplastic transformation [12].

This is the first time that this oncogene was found overexpressed in endometrial carcinoma (30%). It should be emphasized that most oncogenes, as for example the known c-myc and c-erbB2 oncogenes, are found overexpressed in percentages very similar to those we observed.

On analyzing the expression of the mentioned gene by virtual Northern blot at the SAGE (Serial Analysis of Gene Expression) site (http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd=tagsearch&org=Hs&tag=AATCTTTCAT&anchor=NLAII of NCBI), we found MAP3K8 expression present in a great number of tissues, including breast carcinoma, suggesting an eventual role of this gene in that type of tumor as well.

Confirmation of differential results obtained with the membrane through RT-PCR, or another confirmatory method, should always be performed in studies using pools.

This refinement, regarding confirmation of the differential expression was performed individually in a total of ten cases of endometrial adenocarcinoma. The importance of this confirmation is based on two aspects; first the precise finding of the number of cases where there is differential expression; second, it validates the other results of the membrane.

Finally, we conclude that in this study we were able to identify MAP3K8 as a new overexpressed gene in 30% of the cases of endometrial carcinoma, this phenomenon seeming to be correlated with a worse prognosis. However, due to the small number of cases, our results have to be confirmed by studies with a greater number of cases.

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