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Genetic expression profiling of parotid neoplasms by cDNA microarrays

Espressione genica dei tumori parotidei identificata con l'uso di cDNA microarrays

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Key words
Parotid tumours • genetic expression • microarrays

Parole chiave
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Summary

Differentially expressed genes in various benign and malignant salivary gland tumours were identified by use of cDNA microarrays containing 19,000 human expressed sequence tags. Samples were derived from 5 patients with pleomorphic adenoma, 4 with Warthin's tumour, one with clear cell carcinoma, and 2 with mucoepidermoid carcinoma. Tumours were classified by using a subset of 486 genes. Benign Warthin's tumour and pleomorphic adenoma showed very distinctive gene expression patterns. A total of 133 genes differentiated the single malignant clear cell carcinoma from non-tumour salivary glands ($p < 0.01$), whereas only 16 genes separated it from the highly related benign pleomorphic adenoma ($p < 0.01$). A total of 57 cDNAs were associated with mucoepidermoid carcinoma ($p < 0.01$). The results show gene expression alterations common to all tumours and unique to each benign and malignant tumour. The numerous Expressed Sequence Tags of unknown function we identified could also become useful as tumour markers and represent a set of novel tumour-associated genes.

Riassunto

Sono stati identificati con l'uso di cDNA microarrays contenenti 19.000 sequenze geniche, differenti geni espressi in tumori benigni e maligni delle ghiandole salivari. I campioni sono stati ricavati da 5 pazienti affetti da adenoma pleomorfo, 4 da tumori di Warthin, 1 con carcinoma a cellule chiare e 2 con carcinoma muco-epidermoidale. Questi tumori sono stati classificati usando un subset di 486 geni. Il tumore di Warthin e l'adenoma pleomorfo hanno mostrato un pattern di espressione genica estremamente tipico. Il campione di carcinoma a cellule chiare ha dimostrato 133 geni differenti rispetto al tessuto salivare normale ($p < 0.01$), solo 16 geni lo differenziano dall'adenoma pleomorfo ($p < 0.01$), mentre 57 geni erano associati al carcinoma muco epidermoidale ($p < 0.01$). Questi risultati dimostrano che esistono alterazioni dell'espressione genica comuni a tutti i tumori e altre esclusive per i tumori benigni e/o maligni. Sono stati anche identificati numerosi Espressed Sequence Tags, la cui funzionalità è ancora sconosciuta e che potrebbero essere usati come marker tumorali e rappresentare un nuovo gruppo di geni tumore associati.

Introduction

Pleomorphic adenoma is the most common benign tumour of major salivary glands. Cytogenetic analyses have recognized, in addition to a subgroup with normal karyotypes, recurrent chromosomal translocations, with breakpoints at 8q12, 3p21, and 12q13-15 [1] and corresponding to *PLAG1* [2], β-*catenin* (*CTNNB1*) [3], and *HMGIC* [4] genes, respectively. The second most common benign lesion of the salivary glands is Warthin's tumour. It consists of lymphoid stroma and oncocytic epithelium that form cystic or papillary structures. The epithelial component represents the neoplastic proliferation of salivary ducts that have been entrapped within the lymph nodes associated with the salivary gland. Mucoepidermoid carcinoma is the most common malignant, locally invasive tumour of the salivary glands, especially of the parotid gland. Some markers of tumour progression, invasiveness, and prognosis are p21(Kip1), a cyclin-dependent kinase inhibitor, the oncoproteins Bcl-2 and Bax, the tumour suppressor gene product p53, terminal deoxynucleotidyltransferase-mediated nick end labelling staining, and the cell cycle antigen Ki-67 [5 6]. Clear cell carcinoma is a rare form of malignant tumour occurring more often in pleomorphic adenoma. This tumour shows definite areas characteristic of pleomorphic adenoma combined with areas revealing evidence of malignancy. With the technology of cDNA microarrays, it is possible

to make a comparative analysis of mRNA expression of thousands of genes in parallel [7]. Several studies have already demonstrated the usefulness of this technique for identifying novel cancer-related genes and for classifying human cancer at molecular level [8-10]. In this study, a cDNA microarray has been used containing 19,200 different human probes corresponding to different Unigene clusters to characterize benign and malignant salivary gland tumours.

Material and Methods

Patients and tissue specimens

Samples were obtained from surgically resected parotid tissue from 5 patients with pleomorphic adenoma, 4 with Warthin's tumour, one with clear cell carcinoma, and two with mucoepidermoid carcinoma. Informed consent was obtained from patients to use the surgical specimens for research purposes. Part of each sample was frozen in liquid nitrogen and stored at -80°C until RNA extraction, and the remainder was used for histopathological analysis. Tumour paired control, non-tumour, tissues were obtained from a clinically unaffected site and were histologically normal.

Microarray assays

Total RNA was extracted from the biopsies using RNAzol. Ten µg of total RNA were used for each sample. cDNA was synthesized using Superscript II (Life Technologies, Inc.) and amino-allyl dUTP (Sigma). Monoreactive Cy3 and Cy5 esters (Amersham Pharmacia) were used for indirect cDNA labelling. A pool of 8 non-tumour salivary gland RNA was labelled with Cy3 and used as a control against the Cy5-labelled tumour cDNA. Human 19.2 K DNA microarrays, containing ESTs3 corresponding to at least 15,448 different Unigene clusters, were used (Ontario Cancer Institute) [3]. One hundred µl of the sample and control cDNA in DIG Easy hybridization solution (Roche) were used in a sandwich hybridization of the two slides constituting the 19.2 K set at 37 °C overnight. Washing was performed 3 times for 10 min with 1-SSC, 0.1% SDS at 42 °C and 3 times for 5 min with 0.1-SSC at room temperature. Slides were dried by centrifugation for 2 min at 2,000 rpm. Hybridized slides were scanned using the GenePix 4,000A scanner (Axon Instruments, Foster City, CA, USA). Arabidopsis RNA was used as a reference for RNA labelling. Images were analysed using GENEPIX PRO 3.0 (Axon Instruments). Spots showing no signal or obvious defects were accordingly flagged by GENEPIX PRO and excluded from the analysis. Local background was subtracted from the remaining spots, and then spots with fluorescence intensity in both channels of < 1,000 were stringently flagged as absent. The background level for a typical array was of 67.5 (SD, 145.2) for the Cy5 channel and of 182.7 (SD, 305.4) for Cy3 channel. Maximum fluorescence intensity is at about 65,500 in both channels, and arrays were scanned ensuring that < 1% of the total number of spots measured on the slide had median intensities close to maximal. Ratios of net fluorescence from the Cy5-specific channel to the net fluorescence from the Cy3-specific channel were calculated for each spot, representing tumour mRNA expression related to the corresponding non-tumour salivary gland tissue. A normalization factor was estimated from ratios of median by GENEPIX PRO. Cy5:Cy3 expression ratios were then logtransformed (base 2) and normalized, for each different array, by adding the log2 of the respective normalization factor to the log2 of the ratio of medians for each spot within the array, so that the average log-transformed ratio was equal to zero. The threshold for significant RNA expression changes (3.0-fold; i.e, ~ 1.5 on the log2 scale) was established as 3 times the SD of an assay, where the same RNA sample was independently retrotranscribed and labelled with both cyanines. DNA spots present in at least 75% of the arrays (5,681 spotted cDNAs) and with expression ratios higher than the above-defined threshold, in at least one array, were selected for the clustering analysis (4,434 spotted cDNAs). To select the probes that distinguish between two sample groups (e.g, pleomorphic adenoma *versus* Warthin's tumour), we developed a programme performing two statistical tests, t test and log likelihood. Log likelihood was defined as the difference between the mean of each probe in the two groups. To perform multiple t testing correction, the Benjamini and Hochberg procedure, as modified by Storey and Tibshirani [11], was applied. Briefly, it controls the pFDR, defined as the proportion of probes expected to be identified by chance (assuming probes are independent) relative to the total number of probes called significant. This procedure provides a good balance between discovery of significant spots and protection against false positives, because occurrence of the latter is held to a small proportion of the list. A balanced bootstrap analysis with 10,000 permutations was, therefore, applied to the tests to define the number of expected false positives by chance. Expression tables were analysed by using hierarchical clustering, PCA, and similarity searches tools in JExpress v.2.1.5 Variance normalization across the expression table was performed only prior to similarity search to identify probes with a similar expression pattern and was obtained by normalizing the mean and then multiplying each vector by a scalar so that its variance becomes unity.

Table I. Differentially expressed genes in salivary gland tumours.

After t test selection, as explained in Result and Discussion, the gene graphs were individually confirmed by using JExpress. In this table, a selection of genes with the most highly significant t test values is reported. The complete tables can be found at the web site.

Clone	Gene bank	Name	Sample groups[a]	*t* test
Genes up-regulated in salivary gland tumours				
279762	N48348	CEBPD	pleo vs. wart	14.86
297460	W03635	RER1	pleo vs. wart	14.4
298792	W05091	KIAA0105	pleo vs. wart	12.72
259563	N41755	MYO7A	pleo vs. nt	11.96
320698	W32039	DJ37E16.5	pleo vs. nt	11.41
309842	N94626	SNRPD2	pleo vs. wart	11.15
280943	N47576	TSLP	pleo vs. wart	10.71
469566	AA027104	SLC3A1	pleo vs. wart	10.53
281588	N47992	MTP	pleo vs. wart	10.42
114869	T87398	C5	pleo vs. wart	10.37
154604	R55493	PLVAP	pleo vs. nt	10.2
322111	W37664	SLC25A20	pleo vs. wart	10.04
286970	N67453	CDKN1A	pleo vs. wart	9.92
285638	N66473	HNRPA3	pleo vs. nt	9.82
109931	T84450	LOC51651	wart vs. nt	9.59
112468	T85888	DKFZP564O043	pleo vs. nt	9.46
32194	R17332	KIAA0564	pleo vs. nt	9.07
297460	W03635	RER1	pleo vs. nt	8.89
322111	W37664	SLC25A20	pleo vs. nt	8.08
114869	T87395	C5	pleo vs. nt	7.95
503523	AA131279	LMOD1	pleo vs. nt	7.69
212698	H69644	LOC51266	wart vs. nt	7.49
27679	R13006	GDA	wart vs. nt	7.44
162251	H25971	DKFZp434G171	wart vs. nt	7.33
195560	R91819	PIK3R2	wart vs. nt	7.11
116851	T93608	STAM2	wart vs. nt	6.93
146355	R79518	MCAM	wart vs. nt	6.9
41067	R56098	FREQ	wart vs. nt	6.8
207669	H62271	LGP1	wart vs. nt	6.68
109863	T88721	EMP2	pleo vs. wart	5.74
271009	N42817	COX6C	pleo vs. nt	5.72
321607	W32908	FOXO1A	at vs. nt	5.6
308119	N92360	NFATC3	wart vs. nt	5.5
180257	R84726	ADORA1	pleo vs. wart	5.48
201990	R99333	KIAA1733	at vs. nt	5.23
501817	AA127953	HHEX	at vs. nt	5.18
116844	T93783	FLJ13605	wart vs. nt	5.04
278649	N66214	NPEPPS	at vs. nt	4.91
321269	W52940	MIL1	at vs. nt	4.75
446261	AA203732	CHP	at vs. nt	4.7
358848	W94646	ITGAE	wart vs. nt	4.24
109863	T88721	EP2	pleo vs. nt	4.16
Genes down-regulated in salivary gland tumours				
42553	R61223	CAMK2	wart vs. nt	-12.13
242058	H93330	SLC9A3R1	wart vs. nt	-11.59
152489	R62399	MGC11352	wart vs. nt	-9.43

Table. I. Continued

After t test selection, as explained in Result and Discussion, the gene graphs were individually confirmed by using JExpress. In this table, a selection of genes with the most highly significant t test values is reported. The complete tables can be found at the web site.

Clone	Gene bank	Name	Sample groups[a]	t test
33862	R19859	EST	pleo vs. wart	-8.84
46743	H10327	RAP1B	wart vs. nt	-8.74
132026	R24904	PP1665	wart vs. nt	-8.64
29961	R14696	FLJ12438	wart vs. nt	-8.52
111650	T84578	CDC37	wart vs. nt	-8.16
133326	R26844	EST	wart vs. nt	-8.13
265082	N30528	PPARD	wart vs. nt	-8.11
143341	R74281	PPP4C	wart vs. nt	-8.06
289002	N59818	HRIHFB2436	wart vs. nt	-8.04
33862	R19859	EST	pleo vs. nt	-7.98
148698	H12850	EST	pleo vs. nt	-7.97
129794	R16987	SEC8	wart vs. nt	-7.94
207992	H60458	ACOX2	pleo vs. wart	-7.64
488555	AA047136	EST	pleo vs. wart	-7.56
49961	H29294	FLJ20364	pleo vs. wart	-7.54
		Genes up-regulated in salivary gland tumours		
36318	R21064	KIAA0118	pleo vs. nt	-7.52
241224	H91140	FLJ179	pleo vs. wart	-7.32
66513	T66987	EST	pleo vs. wart	-7.28
39241	R51610	EST	pleo vs. nt	-7.14
136872	R36627	11-Oct	pleo vs. wart	-7.06
195560	R91819	PIK3R2	pleo vs. wart	-7.05
236338	H61357	TP53	pleo vs. wart	-7.01
132550	R25899	HNRPD	pleo vs. nt	-6.87
130426	R21806	EST	pleo vs. nt	-6.86
129794	R16987	SEC8	pleo vs. nt	-6.65
380794	AA054151	OXA1L	pleo vs. nt	-6.41
146858	R80974	WEE1	wart vs. nt	-6.26
178468	H47026	MGAT3	pleo vs. nt	-6.21
140878	R67235	BAT1	pleo vs. nt	-6.17
286050	N64281	EST	at vs. nt	-6.01
180257	R84726	ADORA1	wart vs. nt	-5.73
150673	H02088	EST	at vs. nt	-5.57
36318	R21064	KIAA0118	at vs. nt	-5.52
152489	R62399	MGC11352	at vs. nt	-5.49
46743	H10327	RAP18	at vs. nt	-5.41
178468	H47026	MGAT3	at vs. nt	-5.36
380794	AA054151	OXA1L	at vs. nt	-5.35
375693	AA033736	AD037	at vs. nt	-5.28
179383	H50403	FLJ20793	at vs. nt	-5.19
134887	R32270	EMP2	wart vs. nt	-4.61
147839	R81839	TXK	at vs. nt	-3.16

[a] at: all tumours; nt: non-tumor; pleo: pleomorphic adenoma; wart: Warthin's tumour.

Results

We compared the expression profiles of 12 salivary gland tumours and four normal tissue samples by hybridization to microarrays containing 19,200 cDNAs. As the arrays shared a common normal tissue reference made of a pool of 8 non-tumour salivary glands, different from those used as test samples, the samples could be compared to identify gene expression patterns correlated with clinical features of the tumours. We used hierarchical clustering [12] to look at the variation in gene expression between tumours. A clustering analysis was performed by using the selected 4,434 spotted cDNAs, as described in Materials and Methods, corresponding to 2,333 distinct Unigene clusters present in 75% of the arrays and with Cy5:Cy3 expression ratios that varied at least 3.0-fold in at least one array. Using the t test, we then attempted to identify gene expression alterations differentiating the tumours from normal samples. t test selected 713 spotted cDNA probes ($p < 0.01$, average t test significant spots in bootstrap, 91.82; pFDR, 0.05) corresponding to 486 distinct Unigene clusters. Clustering, based on the 486-probe set, correlated very well with the tumour histotypes, with the sole exception of the mucoepidermoid carcinoma (Fig. 1). Warthin's tumours were the most distant branch, with the non-tumour salivary glands in a separate branch from that of the pleomorphic adenoma. The single case of cell carcinoma, developed from a pleomorphic tumour, correctly clustered within the pleomorphic adenoma group. Differentially expressed cancer-associated genes, with the most highly significant t test values [5] are outlined in Table I. Using a similarity search with Euclidean distances on the variance normalized expression table, as obtained using the corresponding JExpress plugin, we identified genes with coherent expression patterns to those of some genes selected by the t test (Fig. 2). Interestingly, among gene subsets that display expression patterns coherent with *FOXO1A*, there are the *PAX1*, homologous to the FOX fused gene described above, a novel Bcl-2 homologue (*MIL1* or *Bcl*-rambo), and the 7b and 7c subunits of cytochrome *c* oxidase (*COX7B*, *COX6C*). Using semiquantitative reverse transcription, we confirmed the expression levels of *OTF1*, *Wee1*, and *EMP2* (data not shown). Furthermore, we identified a number of up- and down-regulated Expressed Sequence Tags (ESTs), which suggest the involvement of a large number of genes that are not yet functionally characterized in salivary gland tumours. Defined subsets of clones that most strongly defined the division of biopsies by histotype were identified using the t test. When we applied this test to compare each tumour to non-tumour samples, we selected 599 cDNA elements differentially expressed ($p < 0.01$, average t test significant spots in bootstrap, 49.95; pFDR, 0.04) in pleomorphic adenoma and 2586 elements ($p < 0.01$, average t test significant spots in bootstrap, 5.73; pFDR, $p < 0.001$) specifically expressed in Warthin's tumours. A total of 1198 clones were differentially expressed ($p < 0.001$, average t test significant spots in bootstrap, 0.13; pFDR, $p <$

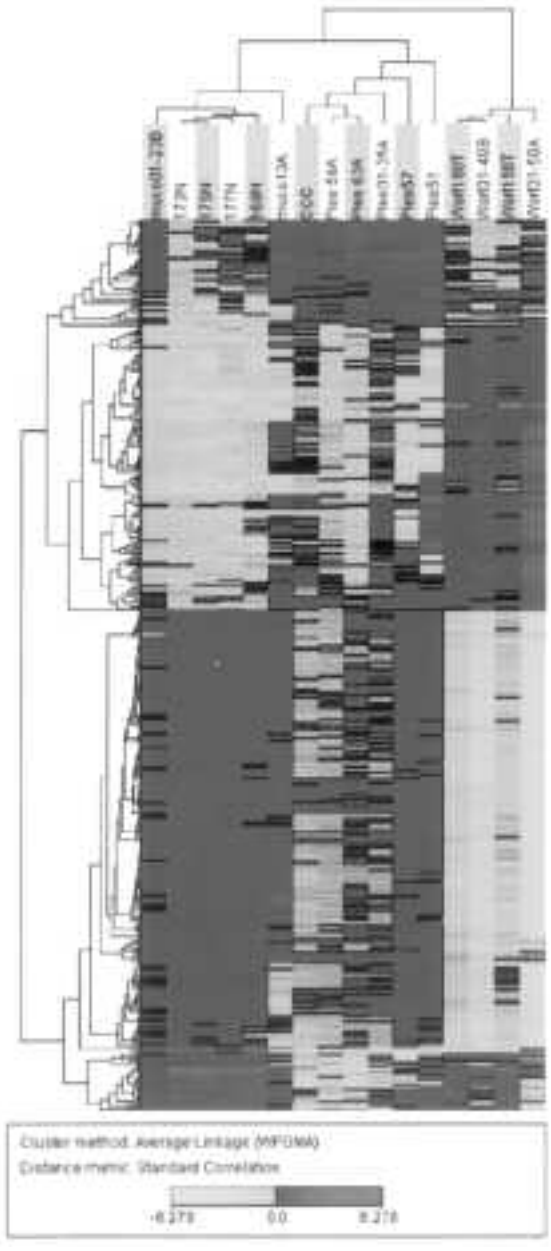

Fig. 1. Clustering of 713 spotted cDNA probes corresponding to 486 Unigene clusters, selected by *t* test, that were differentially expressed in salivary gland tumours ($p < 0.01$, mean *t* test significant spots in bootstrap, 80.4; pFDR, 0.04). Rows, cDNA probes; columns, individual patient samples. Dark grey: probe over-expression; light grey: under-expression.

0.001) in pleomorphic adenoma when compared with Warthin's tumour, of which 479 are over-expressed in Warthin's, whereas 719 are over-expressed in pleomorphic denoma. The specificity of clone expression is shown in Table I. Finally, we selected a subset of clones that most strongly defined the division between malignant and benign lesions. Using the log likelihood test, and as log2 threshold a value of 1.5, corresponding to 3X SD from mean, we identified 16 Unigene clusters differentiating this malignant clear cell carcinoma from its related benign pleomorphic adenoma ($p < 0.01$, as determined by bootstrap distribution). Because, as shown by hierarchical clustering (Fig. 1), Warthin's tumour was very divergent from the other biopsies, we then compared the mucoepidermoid carcinoma to all the other samples after the exclusion of Warthin's tumour samples. Using the t test, we selected 57 cDNA probes ($p < 0.01$). Among the relevant genes repressed in mucoepidermoid carcinoma, there is the *PSG11* member of the carcinoembryonic antigen family and two genes involved in apoptosis, *CIDEB* and *ADORA1*. *CIDEB* or cell death-inducing DFFAlike effector b, is involved in induction of apoptosis by DNA damage [19], and *ADORA1*, adenosine A1 receptor, is involved in induction of apoptosis by extracellular signals. Interestingly, these genes are repressed only in mucoepidermoid carcinoma. *FLJ10355*, which is over-expressed in the mucoepidermoid carcinomas, is also over-expressed in the other malignant tumour clear cell carcinoma and not expressed in the benign tumours. *FLJ10355* has some homology to *Bcl9*, a gene translocated in some B-cell malignancies with abnormalities of 1q21 [18] and with no recognizable protein motifs or significant homologies to any other known proteins.

These data have been partially already published on ref. 20.

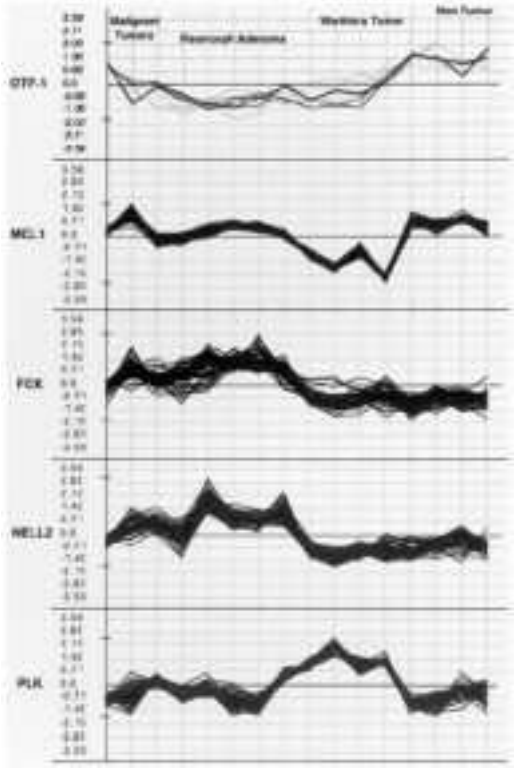

Fig. 2. Clones with expression patterns coherent to prominent tumour-associated genes, selected by using similarity search with Euclidean distances on variance normalized expression patterns.

Discussion and conclusions

The list of up- and down-regulated genes in most salivary gland tumours include known genes involved in neoplasia, members of signal transduction pathways, transcription factors, oncogenes, and tumour suppressor genes, cell cycle regulator, and genes that encode protein involved in cellular metabolism and adhesion. Forkhead box O1A (*FOXO1A*) and *PAX1* are present among up-regulated transcription factors. Fusion of forkhead box O1A with *PAX3*, a *PAX1*-related gene, is associated with solid tumour rhabdomyosarcoma. Over-expression of the fusion protein activates the transcription of anti-apoptotic protein Bcl-XL [12], capable of suppressing cell death pathways by blocking the release of cytochrome *c* from mitochondria and the caspase protease cascade. Cytochrome *c* oxidase down-regulation, alongside cytochrome *c* release to the cytosol, was found to be present in Bax-induced growth arrest [13]. Over-representation of this gene subset suggests that the activation of mechanisms that block apoptosis is a major characteristic of tumour progression in salivary glands. One of the very few genes consistently different in every tumour is *OTF1*, highly expressed in non-tumour salivary glands and under-expressed in all tumours. OTF1 protein binds specifically its POU domain to octamer DNA motifs present in the promoters of several genes and regulates their expression. In addition, OTF1 participates in the cellular response to DNA damage and is involved in the regulation of stress-inducible genes [14]. The identification of a number of up- and down-regulated ESTs suggests the involvement of a large number of genes that remain to be functionally characterized in salivary gland tumours. The many clones associated with Warthin's tumour are probably related to the presence of lymphoid stroma in the affected glands, because some of the selected genes are T-cell specific (i.e, *NFATC3* and *ITGAE,* an integrin preferentially expressed on in-

traepithelial lymphocytes). *Plag1,* a gene that is often rearranged in pleomorphic tumour [19], surprisingly, is significantly over-expressed in Warthin's tumour ($p < 0.01$). In the *Plag1* expression pattern group (Fig. 2), other genes present are: *PLK*1, progranulin, the elevated expression of which confers a transformed phenotype on epithelial cells, including anchorage independence *in vitro* and growth as tumours in nude mice [15]; hypothetical protein FLJ13605 containing a tetra-tricopeptide repeat TPR domain, which forms scaffolds to mediate protein-protein interactions and often the assembly of multiprotein complexes and has a spatial arrangement of α-helices similar to those within 14-3-3 proteins; αL subunit of integrin leukocyte function antigen-1, a membrane glycoprotein that functions in cell-cell adhesion by heterophilic interaction with intercellular adhesion molecule 1; and polo-like serine/threonine kinase PLK1 and TXK, a member of the Tec nonreceptor subfamily of Src protein tyrosine kinases. We identified only 16 Unigene clusters differentiating the probe of malignant clear cell carcinoma from its related benign pleomorphic adenoma. On the contrary, this clear cell carcinoma differed from the Warthin's tumours on account of a much larger number of cDNA elements (1,315), as expected for these two non-related tumours, whereas 133 elements were different from the non-tumour reference pool. Genes differentiating this clear cell carcinoma from its related benign tumour pleomorphic adenoma include myelin transcription factor 1-like, huntingtin, ubiquitin specific protease 9, and *FLJ10355* [5].

In summary, the results show gene expression alterations common to all tumours and unique to each benign and malignant tumour. A larger epidemiological study is necessary to determine whether expression levels of the selected genes can be used as prognostic indicators or whether additional genes need to be included in the analysis. Finally, the numerous *EST*s of unknown function we identified could also become useful as tumour markers and represent a set of novel tumour-associated genes.

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