ORIGINAL ARTICLE

Microarray expression profiling of human dental pulp from single subject

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Abstract

Introduction: Microarray is a recently developed simultaneous analysis of expression patterns of thousand of genes. The aim of this research was to evaluate the expression profile of human healthy dental pulp in order to find the presence of genes activated and encoding for proteins involved in the physiological process of human dental pulp. We report data obtained by analyzing expression profiles of human tooth pulp from single subjects, using an approach based on the amplification of the total RNA.
Methods: Experiments were performed on a high-density array able to analyse about 21,000 oligonucleotide sequences of about 70 bases in duplicate, using an approach based on the amplification of the total RNA from the pulp of a single tooth. Obtained data were analyzed using the S.A.M. system (Significance Analysis of Microarray) and genes were merged according to their molecular functions and biological process by the Onto-Express software.
Results: The microarray analysis revealed 362 genes with specific pulp expression**.** Genes showing significant high expression were classified in genes involved in tooth development, protoncogenes, genes of collagen, DNAse, Metallopeptidases and Growth factors.
Conclusion: We report a microarray analysis, carried out by extraction of total RNA from specimens of healthy human dental pulp tissue. This approach represents a powerful tool in the study of human normal and pathological pulp, allowing minimization of the genetic variability due to the pooling of samples from different individuals.

Expression profiling using the microarray technology represents a powerful approach in the study of gene function in human cells and tissues. Due to the unique ability to analyze simultaneously the expression levels of thousand genes in a single experiment, this approach represents a very high throughput tool in the identification of genes involved in different physiological and pathological conditions and has been largely used in the molecular characterization of different diseases.[1]

Different reports have demonstrated the usefulness of the microarray technology in the study of the gene expression profiles of healthy and carious pulp tooth,

as well as of other dental disorders.[2-4] The identification of genes involved in the molecular response of pulp tissue under carious lesions would be very important for improvement of diagnosis and treatment. However, to generate sufficient amounts of RNA to allow microarray analysis, these experiments are generally carried out by pooling samples from different subjects.[2-4] As a consequence, data reported in the literature provide results representing the average gene expression profile in different patients. Since each individual shows a different genetic background, the use of pooled samples from several subjects increases the inter-patient variability in case-control studies, producing a lower signal-to-noise ratio of the experiment. Moreover, this genetic heterogeneity makes it difficult to discriminate between transcripts that specifically play a role in the disease from those that may be altered due to allelic variation.

To increase the specificity of the results provided by this approach, the use of samples obtained from single individuals would be of great usefulness, allowing for an inter-patient variability to be ruled out affecting the quality of the results.

Previous studies have reported the possibility of obtaining a sufficient amount of RNA to be used in microarrays experiments by means of the total amplification of the RNA obtained from as little as a single cell.[5] This approach allows microarray experiments to be performed when a limited tissue amount is available, avoiding the necessity of pooling RNA from different individuals.

In this research, we report the use of total RNA amplification for the study of gene expression profiles of human dental pulp obtained from single individuals to find the presence of genes activated and encoding for proteins involved in physiological process of human dental pulp.

Methods

Pulp sample

Single samples of human third molar pulp, extracted for orthodontic purposes from four young patients (average age 17yr) in the Chieti University Department of Oral Science, were obtained immediately post-extraction following informed patient consent. Teeth were sliced for the extraction of the tooth pulp within 5 min. The external surfaces were cleaned with clorhexidine 0.2% and all external soft tissue remnants were mechanically removed. A 2-3 mm groove was cut around the teeth using a diamond cutter, avoiding exposure of the pulp. Then teeth were fractured off through the cutting line with a surgical lever, and pulps were removed from the pulp chambers.

RNA extraction and expression profiling

Pulps were placed into RNA later (Ambion, Austin, TX, USA) immediately after removal. RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA concentration and purity were determined by measuring absorbencies at 260 and 280 nm, and a 260:280 ratio of 1.7 was considered acceptable for analysis.

One µg RNA was amplified using the "Amino Allyl MessageAmp™ II aRNA Amplification kit" (Ambion, Austin, TX, USA), able to produce aRNA, containing 5-(3-aminoallyl)-UTP modified nucleotides, able to bind specifically fluorescent dyes Cyanin3 (Cy3) e Cyanin5 (Cy5). The obtained aRNA (5-20 µg) was labelled with Cy3 or Cy5 (Amersham, Pharmacia Biotech, Buckinghamshire, UK) and hybridized on the array. Four different experiments were carried out, by simultaneous hybridization of pulp with a reference RNA composed of testis RNA. This control was chosen because in our laboratory several expression profiles from testis RNA had been previously obtained (personal data), so that this tissue represents in our hand the gold standard as a control for expression profiling.

To correct for differences in dye efficiencies and minimize such a source of bias, we performed dye-

swap experiments, using the assumption that both channels should be equally bright. Analysis was carried out using high-density array containing about 42,658 sequences (21,329 transcripts present in replicates), in form of oligonucleotidic sequences of about 70 bases each, specific for different sequences of the human transcriptome (Micro Cribi, Padova, Italy).

Fluorescent signals were captured by a ScanArray 5000 Packard laser scanning (Packard BioChip Technologies, Billerica, MA) and analyzed using the "ScanArray Express" software. Finally, the obtained data were statistically analyzed using the S.A.M. system (Significance Analysis of Microarray). In each experiment, a 1.4 fold change in the signal of each spot was considered as evidence of a different expression of the specific transcript.

To classify genes according to standardized Gene-Ontology (GO), we used the Onto-Express software (http://vortex.cs.wayne.edu/projects.htm), able to group genes according to their molecular functions and biological process in which are involved.[6] The most significant genes showing high expression were classified and grouped in several clusters according to their function and gene categories.

Results

RNA obtained from single human tooth pulp after amplification was successfully hybridized on high density microarray as demonstrated by analysis of internal control parameter data (Fig. 1).

Among the 42.658 sequences present on the array, more than 17.000 produced a positive signal after hybridization with pulp and testis RNA.

Using the SAM software we performed statistical analysis and shortlisted 546 genes with specific pulp expression, showing a 4:1 ratio as compared with the control tissue (Fig. 2). Among the 546 genes, 184 were "in silico" (genes whose function is still unknown). The remaining 362 genes were merged according to their molecular function and biological process in relation with results of Ontoexpress analysis (Fig. 3). The main molecular functions of the detected genes were: protein binding (19%), metal ion binding (10%), nucleotide binding (9.9%) and zinc ion binding (9.2%). About 17% of the detected genes were of unknown molecular function. Concerning the biological process, signal transduction was the most represented (10.9%), followed by transcription (9.2%) and regulation of transcription (9.2%). Again, a large portion of the detected genes showed unknown biological process (18.8%). Genes significantly overexpressed in dental pulp were than classified in: genes involved in tooth development, protoncogenes, collagen genes, DNAse, metallopeptidases and growth factors. (Table 1).

It is noteworthy that DSPP was very highly expressed in young healthy dental pulp. DSPP is a gene that provides instructions for making a protein called dentin sialophosphoprotein. This protein is cut into three smaller proteins: dentin sialoprotein, dentin glycoprotein, and dentin phosphoprotein. This protein, a component of dentin structure and essential for normal tooth development, is cut into three different proteins: dentin sialoprotein, dentin glycoprotein, and dentin phosphoprotein. Dentin phosphoprotein and dentin glycoprotein are thought to be involved in the normal hardening of collagen, the most abundant protein in dentin. Specifically, these proteins play a role in the deposition of mineral crystals among collagen fibers during tooth formation and mineralization.

Specimen analysis found several genes encoding for collagen, the most abundant protein in the dentin organic matrix. In particular type I and type V collagen resulted the most expressed, but we also found the expression of type III collagen, that has been found in human predentin and odontoblasts[7] matrix production and in human reparative dentin.[8] Several genes encoding for matrix metalloproteineases were also overexpressed in all samples, according to matrix metalloproteinase functions. MMPS endopeptidases cluster genes degrade extracellular matrix proteins, including different collagens, and have a role during dentin matrix organization and mineralization.[9,10] Microarray

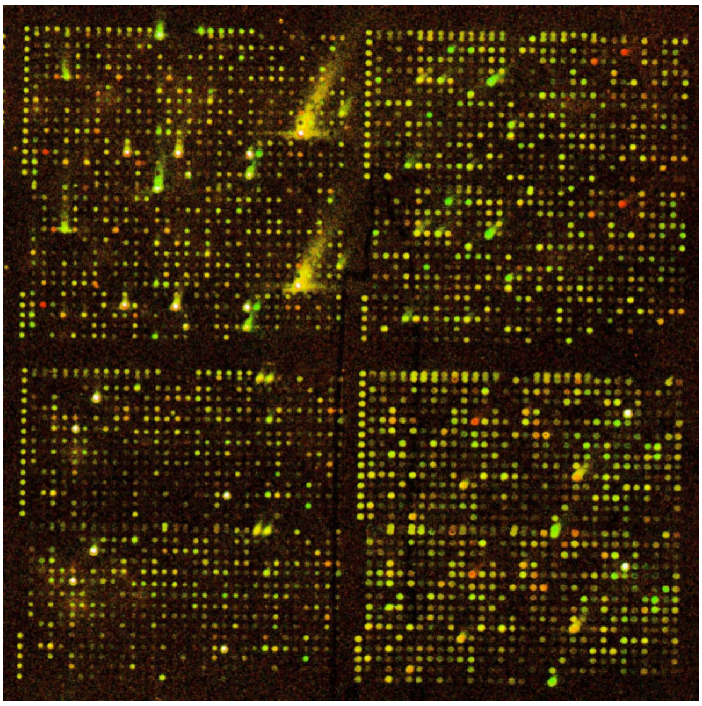

FIGURE 1. ScanArray image of the 21k hybridized slide.

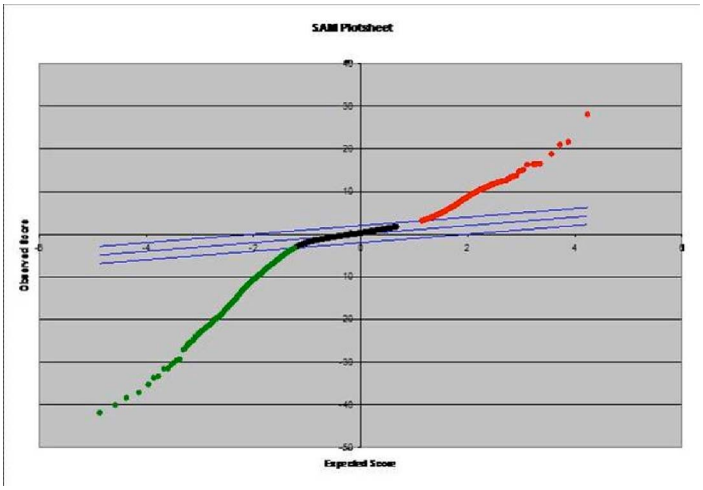

FIGURE 2. Plotter showing SAM analysis is pulp vs testis tissues. Red: human tooth pulp, Green: control tissue (human testis).

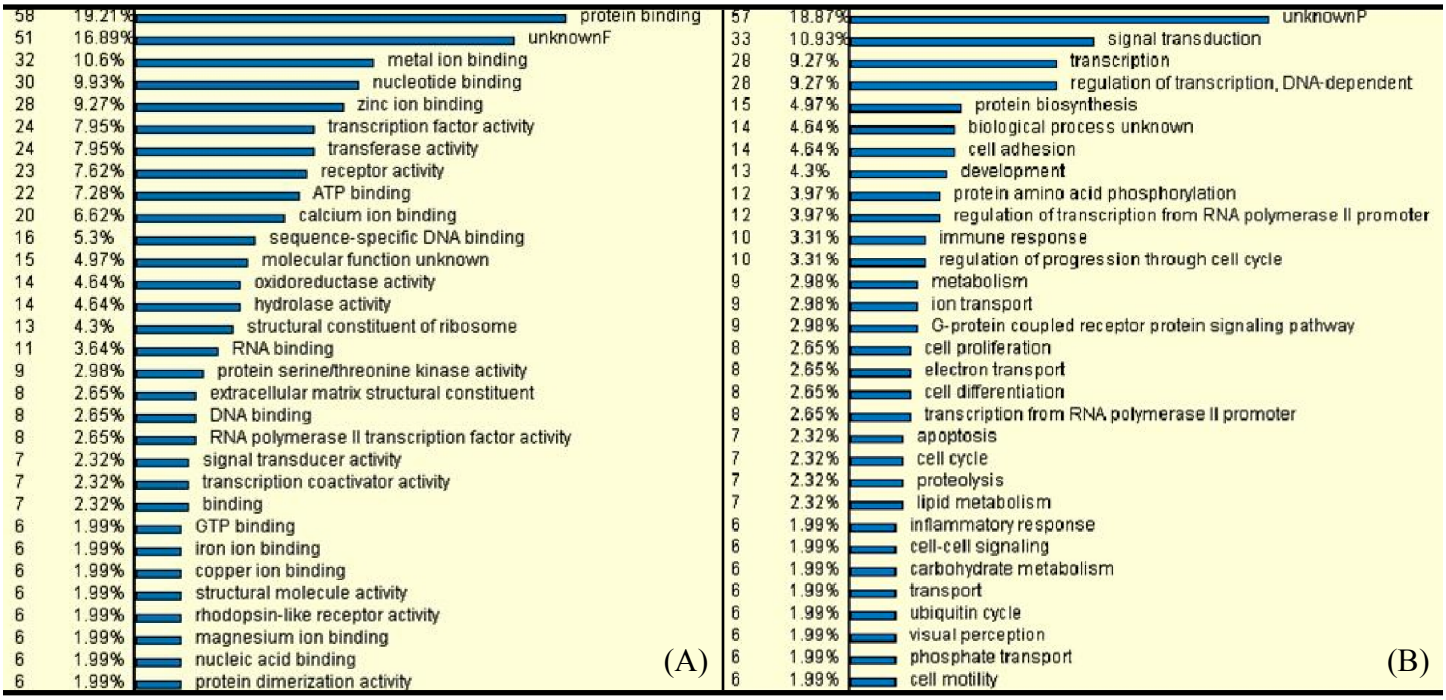

FIGURE 3. Main categories of genes specifically expressed in human tooth pulp grouped on the basis of their molecular function (A) and biologica process (B), as evidenced by Ontoexpress analysis.

TABLE 1. Classification of genes with specific pulp expression after SAM statistical analysis.

Gene categories	Name	Symblol	Gen bank	Cytoband
Genes involved in tooth develpment	Dentine sialophosphoprotein	DSPP	NM_014208	4q21.3
	Osteonectin	ON	NM_003118	5q31.3-q32
	Enamelin	ENAM	NM_031889	4q13.3
	Ameloblastin	AMBN	NM_016519	4q21
	Alkaline phosphatase, liver/bone/kidney	ALPL	NM_000478	1p36.1-p34
	Calbindin 2, 29kDa (calretinin)	CALB2	NM_001740	16q22.2
Protoncogenes	MAS1 oncogene	MAS1	NM_002377	6q25.3-q26
	Jun D proto-oncogene	JUND	NM_005354	19p13.2
	Jun B proto-oncogene	JUNB	NM_002229	19p13.2
	Retinoblastoma-like 2 (p130)	RBL2	NM_005611	16q12.2
	V-ski sarcoma viral oncogene homolog (avian)	SKI	NM_003036	1q22-q24
Collagen	Collagen, type I, alpha 2	COL1A2	NM_000089	7q22.1
	Collagen, type III, alpha 1	COL3A1	NM_000090	2q31
	Collagen, type V, alpha 2	COL5A2	NM_000393	2q14-q32
	Collagen, type V, alpha 3	COL5A3	NM_015719	19p13.2
	Collagen, type IV, alpha 5	COL4A5	NM_000495	Xq22
DNAse	Deoxyribonuclease I-like 1	DNASE1L1	NM_006730	Xq28
Metallopeptidases	ADAM metallopeptidase domain 21	ADAM21	NM_003813	14q24.1
	Matrix metallopeptidase 25	MMP25	NM_022468	16p13.3
	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	MMP2	NM_004530	16q13-q21
	ADAM metallopeptidase domain 8	ADAM8	NM_001109	10q26.3
	ADAM metallopeptidase domain 20	ADAM20	NM_003814	14q24.1
Growth factors	Fibroblast growth factor 12B	FGF12	NM_021032	3q28
	Midkine (neurite growth-promoting factor 2)	MDK	NM_002391	11p11.2
	Pre-B-cell colony enhancing factor 1	PBEF1	NM_005746	7q22.2
	Thymosin, beta 10	TMSB10	NM_021103	2p11.2
	Early growth response 1	TGFB1	NM_000660	19q13.2|19q13.1

analysis showed overexpression of ENAM and AMBN genes encoding for Enamelin and ameloblastin, which represent essential proteins in enamel matrix formation.

The overexpressed gene analysis showed Calbindin 2 (calretinin) and alkaline phosphatase (ALP) protein, genes involved in calcium and phosphate handling synthesized jointly by osteoblasts and odontoblasts. Microarray analysis also revealed several genes encoding for growth factors and protoncogenes that play a fundamental role in tooth development and in fisiological and pathological processes that occur during tooth life.[11] In particular, growth factors like fibroblast growth factor may regulate the expression of collagen and extracellular matrix (ECM)-degrading enzymes, in mature human odontoblasts.[12]

Discussion

Many studies have focused on the ultra structure and molecular aspects of human tissue in physiological and pathological conditions to understand the fine functioning mechanisms. Recently, it has been shown that simultaneous analysis of several genes involved in this processes can be done using the expression profile approach of microarray technique.[13-21]

Microarray technology has been largely used in recent years for the study of expression profiles of different normal and pathological tissues, providing information about the identification of tumour subtypes[13], classification of nodal metastasis in primary larynx squamous cell carcinoma[14], diagnosis and classification of non-Hodgkin's lymphomas[15], diagnosis, treatment, and prevention of infectious diseases[16], identification of genes present in bacteria[17], diagnosis

of allergic diseases[18], gene expression in schizophrenia and related mental disorders[19], and for the identification of genes expressed during mouse tooth development.[20, 21]

Microarray technology has been used in the study of expression profiles of healthy and carious tooth pulp, to identify genes involved during pathogenesis.[2] However, studies so far reported have been carried out by pooling samples from different individuals, in order to generate a sufficient pulp amount to be used in microarray experiments. This approach can decrease the specificity of the obtained expression profiles, since at least a portion of the observed variability can be due to the different genetic background of the investigated patients.[2,3] Variability between hybridizations of different samples was detected in the study of Paakonen[2], suggesting variations among the overall gene expression pattern of individual teeth. In order to reduce this variability, likely due to the different genetic background of the investigated subjects, in our study we carried out experiments in order to demonstrate the microarray approach for producing expression profiles from tooth pulp obtained from a single subject. Using an approach able to perform RNA amplification, our research have demonstrated that successful hybridization can be obtained starting from as little as 100μg of RNA, corresponding to the amount obtained from a single subject. The microarray analysis allowed us to evidence 362 genes with specific pulp expression. Some genes were already found in previous studies (Paakonen et al. 2005). We performed our analysis using high density array so we could analyse several other genes. Although many of the detected transcripts showed unknown function, we found many genes encoding for tooth-specific protein. Dental pulp cells consist mostly of fibroblast, odontoblast and undifferentiated mesenchymal cells. Therefore, our results showed several genes of collagen: type I, V, IV and III and genes encoding for MMPs that have a role in dentin organic matrix organization before mineralization. We also found growth factors that have the ability to regulate the response of the dentin-pulp complex to external irritation. The dentin structure is composed of proteins common to both dentin and bone. Thus, our results showed genes encoding for type I, III, and V collagens, osteonectin (ON) and alkaline phosphatase (ALP) that play a fundamental rule in dentin mineralization. The expression of DSPP, a gene encoding for phosphorylated parent protein that is cleaved post-translationally into three dentin components - dentin sialoprotein (DSP), dentin glycoprotein (DGP), and dentin phosphoprotein (DPP)[22] - was very significant. These proteins are synthesized and secrete by odontoblasts and dental pulp cells to form components of dentin extracellular matrix.[23] The relevance of these genes is in conditions like dentinogenesis imperfecta type II and type III and dentin dysplasia type II [24] that are related to mutations of the DSPP gene.

Overall, the analysis carried out using the Ontoexpress software allowed us to evidence that a large portion of the detected genes showed protein binding as the main molecular function (metal ion binding and zinc ion binding).

In conclusion, in this study we showed the expression profile of healthy tooth pulp demonstrating that RNA amplification is a useful tool for the study of expression profiles starting from samples obtained from a single subject. This approach will allow study of the expression profiles of normal and pathological pulps, without the variability induced by the presence of different genetic backgrounds and to identify specific genes involved in pathological processes.

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