



# Identification of molecular markers related to human alveolar bone cells and pathway analysis in diabetic patients

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**ABSTRACT.** Alveolar bone osteoblasts are widely used in dental and related research. They are easily affected by systemic diseases such as diabetes. However, the mechanism of diabetes-induced alveolar bone absorption remains unclear. This study systematically explored the changes in human alveolar bone cell-related gene expression and biological pathways, which may facilitate the investigation of its mechanism. Alveolar bone osteoblasts isolated from 5 male diabetics and 5 male healthy adults were cultured. Total RNA was extracted from these cells and subjected to gene microarray analysis. Differentially expressed genes were screened, and a gene interaction network was constructed. An enrichment pathway analysis was simultaneously performed on differentially expressed genes to identify the biological pathways associated with changes in the alveolar bone cells of diabetic humans. In total, we identified 147 mRNAs that were differentially expressed in diabetic alveolar bone cells (than in the normal cells; 91 upregulated and 36 downregulated mRNAs). The constructed

co-expression network showed 3 pairs of significantly-expressed genes. High-enrichment pathway analysis identified 8 pathways that were affected by changes in gene expression; three of the significant pathways were related to metabolism (inositol phosphate metabolism, propanoate metabolism, and pyruvate metabolism). Here, we identified a few potential genes and biological pathways for the diagnosis and treatment of alveolar bone cells in diabetic patients.

**Key words:** Diabetes; Alveolar bone cell; Biological pathway; Bioinformatics

## INTRODUCTION

Bone tissue has a strong regenerative capacity. Bone reshaping originates from osteoclast absorption and is maintained by osteoblast formation (Vestergaard, 2014). *In vitro* cultured bone cells provide valuable information regarding bone growth and metabolism, and for the treatment of bone disease. The *in vitro* osteoblast model is the most actively researched bone cell model (Grey, 2015). *In vitro* alveolar bone osteoblast culture is widely used in the study of teeth and tooth-related diseases. Alveolar bone is an active region of bone metabolism in the body, and is easily affected by systemic diseases, such as diabetes mellitus (DM) (Sargent, 2014). Previous research studies have indicated that the reduced infection resistance of local tissues aggravates periodontal inflammation, which in turn results in alveolar bone inflammatory absorption. However, the specific mechanism of diabetes-induced alveolar bone resorption remains to be elucidated (Khosravi and Trackman, 2014).

Owing to the complexity of the disease, specifically with respect to the gene-gene interactions governing the disease, recent studies have focused on elucidating the gene expression via network analysis. The co-expression network is the best among the various networks (analysis techniques) to indicate the correlation between genes, since the network is constructed based on changes in the gene expression and direction of regulation. The empirical Bayesian co-expression analysis method is the best method (among all available methods) to identify different groups without genome condition number limitation, while controlling the false discovery rate (FDR) (Dawson and Kendzioriski, 2012). Broët et al. (2002) confirmed that the empirical Bayesian method can be used to identify changes in the microarray gene expression and co-expression analysis (Broët et al., 2002). Therefore, the empirical Bayesian method was used in this study to build a gene expression network, and to explore the relationship between differential gene expression and pathway analysis in alveolar bone osteoblasts obtained from diabetes patients.

Primary *in vitro* alveolar bone osteoblast cultures were prepared in this study; these were subjected to high-throughput gene expression analysis, to determine the molecular mechanism of the effect of diabetes on the biological character of, and pathological changes in alveolar bone osteoblasts. In addition, the microarray characteristics of primary culture models were investigated, and differentially expressed genes between diabetic patients and normal controls were compared, to further select a local implantable drug delivery system.

## MATERIAL AND METHODS

### Alveolar bone acquisition

Alveolar bone debris was obtained from five male type-2 diabetic patients and normal controls with tooth loss and treated with oral planting. The ages of the enrolled subjects varied between 45 and 50, and the DM type 2 (DM2) patients had at least 5 years of diabetes history. Random blood glucose and glycosylated hemoglobin levels were determined to be 11-15 mM/L and 10-15% in diabetic patients without glucose control, and 4.5-5.9 mM/L and 5-6% in healthy subjects. The blood glucose levels were controlled in the DM2 patients; the resulting fasting blood glucose and glycosylated hemoglobin levels were 5-6 mM/L and 5-6%, respectively. Informed consent was obtained from all patients prior to the surgery. Enough alveolar bone mass was reserved to guarantee plantation. The bone fragments obtained on the pioneer and twist drills were collected for further experiments.

### Osteoblast cultivation

The collected bone debris was flushed thrice with D-Hanks buffer (Nanjing Shenbeijia Biotechnology Co., Ltd.) supplemented with 100 mg/L penicillin-streptomycin, and subsequently centrifuged twice at 300 g for 5 min. The supernatant was removed, and the pellet was cultured on a dish coated with a small amount (1100 mg/L) of fetal bovine serum. Bone debris was moistened with low-sugar Dulbecco's modified Eagle's medium (Gibco), and maintained in an incubator at 37°C and 5% CO<sub>2</sub>. Complete medium was added to this after 3 hours, and the medium changed every 3 days. The cells were collected upon covering approximately 80% of the bottom (area) of the culture dish.

### RNA extraction and purification

RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), according to the standard operating procedure provided by the manufacturer. The sample was ground with liquid nitrogen, TRIzol reagent was added for 5-10 minutes, and the chloroform was subsequently added at room temperature for 15 min. This was centrifuged for 15 min, and the obtained sample was incubated with isopropyl alcohol for 10 min. Another round of centrifugation for 10 min was carried out and total RNA was extracted by the addition of DEPC-treated water. The extracted RNA was quantified by electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA samples were then purified using the RNeasy micro kit (Qiagen, Venlo, Netherlands) and RNase-free DNase kit (Qiagen, Hilden, Germany). The obtained RNA samples were reverse transcribed to obtain cDNA by the RT kit (Bio-Rad), and the sample was ultimately scanned after hybridization.

### Gene microarray

Microarray profiling of the synthesized mRNA was performed using the HumanGene U133 plus 2.0 Arrays (HumanGene, Santa Clara, CA, USA) according to manufacturer protocols. The Affymetrix array cassette was subjected to standard staining, washing, and scanning. Feature extraction was performed using the Affymetrix Command Console software (Barrett

et al., 2013). The raw data was processed in the following sequence: background detection was followed by RMA global background correlation, quantile normalization, median estimation, and log2-transformation, using the mRNA QC software tool (Affymetrix).

The Limma package was used to treat the extracted data, and the significance was detected using the *t*-test.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{S_1^2 / n_1 + S_2^2 / n_2}} \quad (\text{Equation 01})$$

Mean:

$$\bar{X}_i = \sum_{j=1}^{n_i} X_{ij} / n_i \quad (\text{Equation 02})$$

Deviation:

$$S_i^2 = \frac{1}{n_i - 1} \sum_{j=1}^{n_i} (X_{ij} - \bar{X}_i)^2 \quad (\text{Equation 03})$$

Here,  $n_i$  represents the repeat time of the experiment,  $X_{ij}$  denotes a certain gene expression level for condition  $i$  and the  $j$  time repeat. The P value was obtained based on the statistical value of  $t$ . P value less than 0.05 was considered to be a statistically significant.

The Benjamini and Hochberg algorithm (Benjamini and Hochberg, 1995) was used to correct the sample P value for multiple testing FDR (threshold value, 0.05); genes exhibiting P values < 0.01 were treated as differentially expressed genes. The FDR correction was calculated as follows:

$$FDR = E(V / R) \quad (\text{Equation 04})$$

where,  $V$  denotes the unobservable random variables and  $R$  indicates observable random variables.

### Co-expression network

Gene co-expression networks were built using the empirical Bayesian approach. The log2 value was applied for background standardization of the expression matrix  $m \times n$  ( $m$  denotes the number of genes at a specific condition and  $n$  indicates the total number of genes in the microarray), resulting in the matrix  $X$ . " $n$ " indicated the length of the condition sequence, whose value should vary between 1 and  $K$  ( $K$  indicates the total number of conditions). The differential co-expression/identical co-expression (EC/DC) grade was defined.

The genes were subjected to intra-class correlation calculation, using the equation  $p = m * (m - 1) / 2$ , and the correlation matrix  $D$  ( $p \times K$ ). The hyper-parameters were subjected to an initial treatment to ensure consistency of the transformed  $D$  with the components of the standard hybrid model; the posterior probability of DC production was estimated. The match degree of the selected model and database was tested. In case of good model matching, the model was provided with an ideal soft threshold value. However, soft threshold might result in higher FDR; therefore, the FDR was set to  $\leq 0.05$ .

## Pathway analysis

The significant pathways affected by the differential genes were identified by pathway analysis, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Enrichment analysis was also used to select significant pathways (Grossgaluser and Vetterli, 2003). The enrichment analysis was performed using the following equation

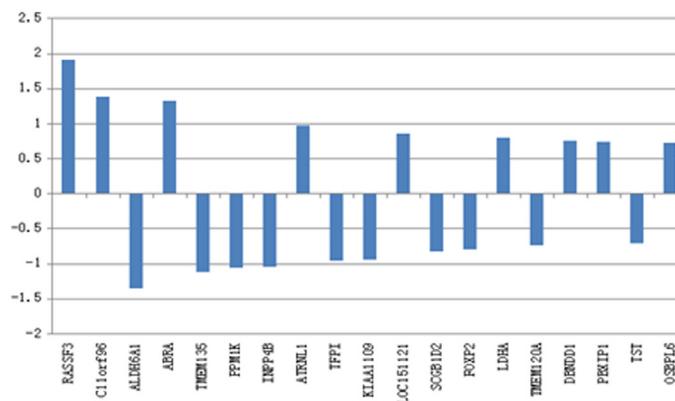
$$P = \frac{\binom{M}{k} \binom{N-M}{n-k}}{\binom{N}{n}} \quad (\text{Equation 05})$$

where,  $N$  is the total number of genes in the microarray,  $n$  denotes the number of differential genes within a particular category,  $M$  indicates the number of genes in  $N$ , that pertain to a specific biological process, and  $k$  denotes the number of genes in  $n$  that pertain to a specific process.  $P$  is the degree of confidence of enrichment of differentially expressed genes to a specific biological process. A threshold  $P$  value  $\leq 0.05$  was set for significance. Functional enrichment analysis was performed using the DAVID software platform (Huang Da et al., 2008), which is a comprehensive online analysis tool that facilitates pathway enrichment analysis to mine the statistically significant differentially expressed genes or co-expressed genes. This software can be used to quickly determine gene enrichment pathways and tentative biological significance. DAVID can be used to identify signaling pathways with differentially expressed genes.

## RESULTS

### Differentially expressed genes

We identified a total of 147 differentially-expressed mRNAs in alveolar bone cells obtained from diabetics (91 upregulated and 56 downregulated mRNAs) by the Student  $t$ -test. Figure 1 lists 20 genes with the highest significance, including *RASSF3*, *C11orf96*, *ALDH6A1*, *ABRA*, *TMEM135*, *PPM1K*, *INPP4B*, *ATRNL1*, *TFPI*, *KIAA1109*, *LOC151121*, *SCGB1D2*, *FOXP2*, *LDHA*, *TMEM20A*, *DBNDD1*, *PBXIP1*, and *TST*.



**Figure 1.** Differentially expressed genes in alveolar bone cells of diabetic and healthy control patients.

## Pathway analysis

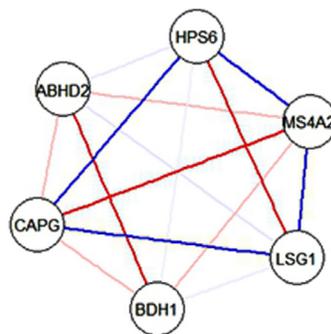
Significant pathways with P values < 0.05 were screened via KEGG pathway enrichment analysis (Table 1). The identified 147 differentially expressed genes were mainly enriched in 8 pathways, majority of which were metabolic pathways. The metabolic pathways with the highest level of significance were inositol phosphate metabolism, propanoate metabolism, and pyruvate metabolism.

**Table 1.** KEGG pathway analysis.

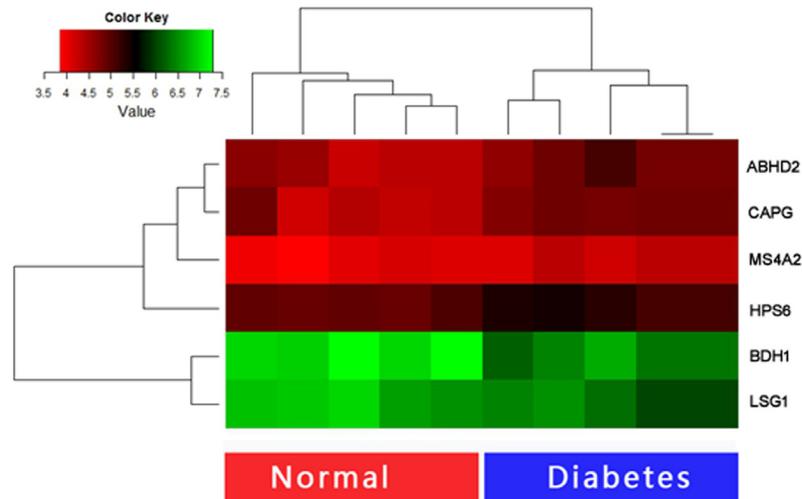
No	Pathway	P value
1	Inositol phosphate metabolism	0.0055
2	Propanoate metabolism	0.0204
3	Pyruvate metabolism	0.0248
4	Valine, leucine and isoleucine degradation	0.0269
5	Non-small cell lung cancer	0.0320
6	Glycolysis/Gluconeogenesis	0.0349
7	Complement and coagulation cascades	0.0390
8	ErbB signaling pathway	0.0464

## Co-expression of differentially expressed genes

Co-expression networks were constructed based on the dynamic changes in gene expression values, regulation relationships, and regulation direction (Stuart et al., 2003). Co-expression analyses can reveal the interaction of genes with each other, and the changes effected by these genes in the disease. Three pairs of genes (*ABHD2* and *BDH1*, *MS4A2* and *CAPG*, *LSG1* and *HPS6*) showed significant differences (Figure 2). The white dots in Figure 2 represent genes, the red line indicates positive co-expression, blue line denotes negative co-expression, and the deeper colors indicated a higher degree of co-expression. The results indicated that the gene pairs exhibited identical trends in both diabetes and healthy control alveolar cells. Meanwhile, there was change in the co-expression relationship in the diabetes group, compared to the normal group. The 6 differentially expressed genes were further subjected to a cluster analysis (Figure 3). Each row in Figure 3 represents a gene, red indicates negative expression, green denotes positive expression, values closer to the green or red were indicative of higher or lower expression, and black represented a low degree of expression. The expression of *BDH1* and *LSG1* was significantly different between the diabetes patients and healthy controls.



**Figure 2.** Differences in the co-expression network of alveolar bone cells in diabetes and healthy controls.



**Figure 3.** Heat map of differentially expressed genes in diabetic patients than those in the healthy control subjects.

## DISCUSSION

Osteoblasts are derived from pluripotent bone marrow stromal cells. They are the major functional cell involved in bone matrix synthesis, secretion, and mineralization. Alveolar bone osteoblasts play an important role in bone matrix collagen formation and osteoclast resorption control, and facilitate the maintenance of balance in the bone metabolism. However, such a balance is vulnerable to the effects of systemic diseases, especially diabetes (Fiorellini and Nevins, 2000). Diabetes influences osteoblasts via two mechanisms: reduced insulin secretion, which may lead to reduction in osteoblast number and bone matrix synthesis; continuous high blood glucose levels, which would restrict osteoblast differentiation, which in turn delays bone deposition and mineralization (Santana et al., 2003; Mellado-Valero et al., 2007). Therefore, it is important to study the biological characteristics of osteoblasts in DM patients. At present, very little scientific literature is available regarding alveolar bone osteoblast-related gene expression and enriched pathway analysis in diabetes patients.

Our study revealed the presence of 147 differentially expressed genes in alveolar bone osteoblasts in DM patients, compared to the normal control subjects (91 upregulated and 36 downregulated mRNA). The co-expression network developed based on the empirical Bayesian approach revealed six differentially-expressed gene pairs. Functional annotation and classification of genes using the DAVID software suggested that these differentially-expressed genes affected eight pathways that were mainly related to metabolism.

Pathway analysis mainly enriched the metabolic related pathways. Diabetes is a metabolic disease with many complications that seriously influence the quality of life of the patients. Therefore, it is important to attempt the early detection and prevention of diabetes. The use of metabolic methods for early classification and diagnosis of diabetes has become an important focal area for diabetes research, and is playing an increasingly important role in diabetes diagnosis (Bain et al., 2009). Studies have shown that diabetes caused carbohydrate metabolic disorder, can reduce bone formation, and decrease bone mineral density. Mean-

while, diabetes can also further influence protein and fat metabolism (Xu et al., 2014). For example, Yi et al. (2006) obtained valuable information regarding biomarkers through the pattern recognition in DM patients and healthy controls (YiHe et al., 2006). In this experiment, diabetes was found to be mainly associated with carbohydrate metabolism, suggesting that glyco-metabolism plays an important role in osteoblast formation and function. This also provided credibility to the enrichment results.

Inositol phosphate, propionic acid, and pyruvic acid are the intermediates of glyco-metabolism under normal physiological conditions. Diabetes is known to cause disorders in the glucose metabolism, impacting a series of metabolic processes, and eventually leading to multiple metabolic disorders (Garg et al., 2014; Lopez et al., 2014). The pentose phosphate pathway is a major pathway for glucose metabolism. This pathway provides NADPH oxidase to maintain the reduced state of glutathione, which is closely related to diabetes (Eid et al., 2009). Meanwhile, pyruvic acid is inoxidizable, which promotes tissue glycolysis, inhibits sorbitol synthesis, improve lens energy metabolism, and delays the occurrence of diabetes (Grey, 2015).

*RASSF3* showed the most significant difference among the 147 differentially expressed genes. *RASSF3* contains five exons, and encodes a 28.6-kDa protein composed of 247 amino acids. The carboxyl end of the *RASSF3* protein contains a highly conserved Ras-related domain (Tommasi et al., 2002). *RASSF3* is widely expressed in normal tissues, and is down-regulated in lung, ovarian, and colorectal cancer tissues (Tommasi et al., 2002; Jacquemart et al., 2009). The downregulation of *RASSF3* resulted in hypermethylation of the promoter region, which in turn accelerates tumor growth by inhibiting apoptosis (Jacquemart et al., 2009). For example, *RASSF3* hypermethylation may result in gene silencing, which might be an important early indicator of pituitary adenoma (Peng et al., 2013). These suggested that *RASSF3* is an important regulatory protein. So far, there has been no report regarding the role of *RASSF3* in diabetes. Our results revealed that *RASSF3* is upregulated in diabetic alveolar bone cells, which may result in an increase in alveolar bone cell apoptosis and a decrease in cell proliferation.

In summary, 147 differentially-expressed genes were identified. The enrichment pathway analysis indicated that metabolic reactions were mainly affected by these genes. Therefore, the results of this study identified a few potential gene- and biological pathway-targets for the diagnosis and treatment of alveolar bone cells in diabetic patients. Metabolic pathways can be used to analyze and judge the proliferative ability of diabetic alveolar bone cells. Therefore, this study lays a foundation for selection of future local implantable drug delivery systems.

### Conflicts of interest

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

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### REFERENCES

Bain JR, Stevens RD, Wenner BR, Ilkayeva O, et al. (2009). Metabolomics applied to diabetes research moving from information to knowledge. *Diabetes* 58: 2429-2443.

- Barrett T, Wilhite SE, Ledoux PL, Evangelista C, et al. (2013). NCBI GEO: archive for functional genomics data sets-update. *Nucleic Acids Res.* 41: D991-D995.
- Benjamini Y and Hochberg Y (1995). Controlling the false discovery rate-a practical and powerful approach to multiple testing. *J. R. Statist. Soc.* 57: 289-300.
- Broët P, Richardson S and Radvanyi F (2002). Bayesian hierarchical model for identifying changes in gene expression from microarray experiments. *J. Comput. Biol.* 9: 671-683.
- Huang Da W, Sherman BT and Lempicki RA (2008). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4: 44-57.
- Dawson JA and Kendziorski C (2012). An empirical Bayesian approach for identifying differential coexpression in high-throughput experiments. *Biometrics.* 68: 455-465.
- Eid AA, Gorin Y, Fagg BM, Maalouf R, et al. (2009). Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases. *Diabetes.* 58: 1201-1211.
- Fiorellini JP and Nevins ML (2000). Dental implant considerations in the diabetic patient. *Periodontology 2000* 23: 73-77.
- Garg M, Mehra P and Bansal DD (2014). Hormonal imbalance and disturbances in carbohydrate metabolism associated with chronic feeding of high sucrose low magnesium diet in weanling male wistar rats. *Mol. Cell. Biochem.* 389: 35-41.
- Grey A (2015). Diabetes medications and bone. *Curr. Osteoporos. Rep.* 13: 35-40.
- Grossglauser M and Vetterli M (2003). INFOCOM 2003. Twenty-Second Annual Joint Conference of the IEEE Computer and Communications. IEEE Societies, 1954-1964.
- Jacquemart IC, Springs AE and Chen WY (2009). RASSF3 is responsible in part for resistance to mammary tumor development in neu transgenic mice. *Int J Oncol.* 34: 517-528.
- Khosravi R and Trackman PC (2014). Diabetes-induced fibrotic matrix inhibits intramembranous bone healing. *J. Cell Commun. Signal* 9: 19-26.
- Lopez LM, Grimes DA and Schulz KF (2014). Steroidal contraceptives: effect on carbohydrate metabolism in women without diabetes mellitus. *Cochrane Database Syst. Rev.* 4: CD006133
- Mellado-Valero A, Ferrer-García JC, Herrera Ballester A and Labaig Rueda C (2007). Effects of diabetes on the osseointegration of dental implants. *Med. Oral Patol. Oral Cir. Bucal.* 12: E38-E43.
- Peng H, Liu H, Zhao S, Wu J, et al. (2013). Silencing of RASSF3 by DNA hypermethylation is associated with tumorigenesis in somatotroph adenomas. *PLoS One* 8: e59024.
- Santana RB, Xu L, Chase HB, Amar S, et al. (2003). A role for advanced glycation end products in diminished bone healing in type 1 diabetes. *Diabetes* 52: 1502-1510.
- Sargent J (2014). Diabetes: Functional impairment of bone marrow progenitor cells in diabetes mellitus. *Nat. Rev. Endocrinol.* 10: 379.
- Stuart JM, Segal E, Koller D and Kim SK (2003). A gene-coexpression network for global discovery of conserved genetic modules. *Science* 302: 249-255.
- Tommasi S, Dammann R, Jin SG, Zhang XF, et al. (2002). RASSF3 and NORE1: identification and cloning of two human homologues of the putative tumor suppressor gene *RASSF1*. *Oncogene* 21: 2713-2720.
- Vestergaard P (2014). Diabetes and bone fracture: risk factors for old and young. *Diabetologia* 57: 2007-2008.
- Xu F, Dong Y, Huang X, Li M, et al. (2014). Decreased osteoclastogenesis, osteoblastogenesis and low bone mass in a mouse model of type 2 diabetes. *Mol. Med. Rep.* 10: 1935-1941.
- Yi LZ, He J, Liang YZ, Yuan DL, et al. (2006). Plasma fatty acid metabolic profiling and biomarkers of type 2 diabetes mellitus based on GC/MS and PLS-LDA. *FEBS Lett.* 580: 6837-6845.