



DEREGULATION OF CANCER-ASSOCIATED GENES IN ODONTOGENIC CYSTS

ABSTRACT




Objectives: The aim of the present study was to demonstrate the key role of differential expression levels of RB1, TP53, XIAP, BCL2 AIFM3, BAX, CASP3 and CASP9 genes in odontogenic cysts.

Materials and Methods: A total number of 15 patients who diagnosed with odontogenic cyst were enrolled for the present study. For the quantitative gene expression analysis, cyst and adjacent gingival healthy tissues of patients were collected during surgical assessments. Quantitative analysis of gene expression levels RB1, TP53, XIAP, BCL2 AIFM3, BAX, CASP3 and CASP9 were achieved real-time PCR method. For the optimization of gene expression levels GAPDH reference gene was used.

Results: Expression of both RB1 and TP53 genes were markedly diminished in odontogenic cysts tissues as compared to healthy tissues ($p<0.05$). Likewise, levels of CASP3 and CASP9 genes were found to be significantly reduced in odontogenic cysts tissues compared to healthy tissues ($p<0.05$). In contrast, expression levels of XIAP was significantly elevated ($p<0.05$). Although BCL2, AIFM3, and BAX genes were also differentially expressed in odontogenic cysts tissues, these variations were statistically insignificant ($p>0.05$).

Conclusions: The findings of the present study indicates that RB1, TP53, XIAP, CASP3 and CASP9 genes might have chief roles in formation odontogenic cysts and responsible for the increased cell proliferation in these tissues.

Keywords: Gene expression, odontogenic cysts, tumor suppressor genes.

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INTRODUCTION

Cysts are pathological cavities which are surrounded by epithelial connective tissue capsule filled with fluid or semi-fluid material. Cysts are most commonly seen in jaws compared to other parts of the body, as epithelial debris in these regions is more abundant than other parts of the body.¹ Radicular cysts are the most frequently encountered odontogenic cysts and originate from a previously formed apical granuloma in the apical region of a necrotic tooth. These cysts are formed by the proliferation of malassez epithelial residues.^{2,3} Dentigerous cysts are cysts that surround the crown of the unerupted or partially erupted teeth. After the formation of the tooth crown, fluid accumulation between tooth enamel epithelium and the tooth crown and additionally proliferation of epithelial islets in the dental follicle wall leads to cyst formation. Approximately 24% of odontogenic cysts are dentigerous cysts and second most common odontogenic cysts seen after radicular cysts.^{4,5} Odontogenic keratocysts, which originates from odontogenic epithelial wastes and the basal layer of the oral epithelium, are considered to be the most aggressive odontogenic cyst and more likely to transform into a malignancy due to local aggressive course, high residual rate and invasion to surrounding tissues.⁶⁻⁹

Besides, molecular pathogenesis of carcinogenesis involves the accumulation of mutations in oncogenes and tumor suppressor genes and these genetic aberrations are the very early sign of a malignant transformation.¹⁰ In consequence, oncogenes and tumor suppressor genes are the crucial components that drive the formation of cancers.^{10,11} Mutations can lead to hyper- or hypo-activity of an oncogene and tumor suppressor gene, respectively. So far, several genes with oncogenic and tumor suppressor functions have been identified to be associated with the development and progression of human cancers.^{10,11} Chiefly, deregulation of these genes have been shown to be well-correlated with poor prognosis and clinicopathological characteristics of patients and experimentally associated with the

increased proliferation, invasion, migration and metastasis of cancer cells in vitro.^{10,11}

Moreover, accumulating body of evidence suggest that several cancer biomarkers were revealed to be deregulated in odontogenic cysts. Particularly, immunohistochemical analysis of Cyclin D1, BCL2, PCNA, P53^{12,13}, COX-2¹⁴, Ki-67, P63^{15,16} HIF-1 α and Caspase 3¹⁷ proteins revealed that these proteins markedly involved in pathobiology of odontogenic cysts and associated with the clinicopathological characteristics of patients. Nevertheless, these findings were limited to immunohistochemistry method and a few numbers of markers were investigated. Apart from these studies, we used quantitative PCR approach which is a cost-effective and less time-consuming method enabling rapid quantitative analysis gene expression levels.

Consequently, in this particular study, our aim was to elucidate the roles of differential expression levels of RB1 (Retinoblastoma 1), TP53 (Tumor Protein P53), XIAP (X-linked inhibitor of apoptosis), AIFM3 (Apoptosis inducing factor, mitochondria associated 3), BAX (BCL2 associated X, apoptosis regulator), CASP3 (Caspase 3) and CASP9 (Caspase 3) genes, which have been known to be associated with the formation and progression of several types of cancers, in odontogenic cysts of radicular, dentigerous and odontogenic keratocyst epithelium and their association with local aggressive behavior and osteolytic properties of these cysts. In particular, expression levels of RB1, TP53, XIAP, BCL2 AIFM3, BAX, CASP3 and CASP9 genes were measured by Real-Time Polymerase Chain Reaction (PCR) method.

MATERIALS AND METHODS

Study population and sample collection

Total numbers of 15 patients who admitted to clinic of Department of Oral and Maxillofacial Surgery with the various symptoms (pain, swelling, etc.) and diagnosed with odontogenic cyst were enrolled in the study. Clinical and demographical features of age, gender, anatomic localization of tooth and pathological diagnosis of patients were shown in Table 1. Among these patients, 4 (26.6%) were odontogenic keratocyst,

3 (20%) were dentigerous cyst and 8 of them were 8 (53.4%) radicular cyst (Table 1). Patients included in the study were subjected to routine examination and panoramic radiographs were obtained. Exclusion criteria for the study was

presence of systemic disease, infection in the operation area, presence of systemic viral, fungal, or bacterial infection, sensitivity to drugs and anesthetics, cigarette or alcohol addiction, pregnancy or lactation.

Table 1. Demographic and clinicopathological characteristics of patients

Parameters	n=15 (%)	
Age	34.8± 23.99	
Gender	Male	6 (40.0)
	Female	9 (60.0)
Localization of Tooth	Mandibula right anterior	1 (6.67)
	Mandibula left posterior	2 (13.3)
	Maxilla right anterior	3 (20)
	Maxilla left anterior	6 (40)
Pathological diagnosis	Mandibula right posterior	3 (20)
	Odontogenic keratocyst	4 (26.6)
	Dentigerous cyst	3 (20)
	Radicular cyst	8 (53.4)

For the study, tissues and adjacent healthy tissues of patients were collected during surgical operation. Collected tissue samples of patients were stored at -80 °C for expression analysis. Also, every tissues sample were subjected to pathological examination prior to inclusion in the study. A written informed consent was obtained from all individuals participated in the study.

Isolation of total RNA from tissue samples

GeneJET RNA Purification Kit (Thermo Fisher Scientific Inc., Wilmington, USA) was used for the isolation of total RNA from tissue samples. Briefly, 30 mg of tissue samples were homogenized in 300 µl lysis buffer containing β-mercaptoethanol (Sigma-Aldrich, MO, USA) by using TissueLyser LT (QIAGEN Sample & Assay Technologies, Germany) and instructions of the manufacturer were followed to obtain RNA. To ensure any RNase contamination RNase AWAY Decontamination Reagent (Thermo Fisher Scientific Inc., Wilmington, USA) was used. Quantities and concentrations of isolated RNA samples were measured by using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) and stored -80°C in equal aliquots.

Synthesis of complementary DNA from RNA samples

Complementary DNA was synthesized by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Wilmington, USA). Briefly, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor, 1 µl of 10 mM dNTP Mix, 1 µl of RevertAid Reverse Transcriptase, and 1 µl of Random Hexamer and variable amounts of ddH2O and RNA samples were mixed and distributed to 0.2 ml PCR tubes. The prepared PCR mix was subjected to following thermal cycling protocol; 5 min at 25°C, 60 min at 42°C and for the termination of reaction 5 min at 70°C. Subsequently, samples were placed on ice block and transferred to -80°C until further application.

Primer Design and Analysis of gene expression by Real-Time PCR

Appropriate expression primers were designed using the "Primer-blast" interface of National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to analyze the expression of the genes of interest. The primers and their properties used in the analysis of mRNA expressions. For the analysis of the expressions of RB1, TP53, XIAP, BCL2 AIFM3, BAX, CASP3 and CASP9 genes, we used RealQ Plus 2x Master Mix Green without ROX™ (Ampliqon PCR Enzymes & Reagents, Odense M, Denmark). Briefly, for each reaction

12.5 μ l of RealQ Plus 2x Master Mix, 0.5 μ l of Forward primer, 0.5 μ l of Reverse primer, 2 μ l of cDNA and 9.5 μ l of PCR-grade H₂O was used. Real-time PCR reactions were carried out in Rotor-Gene Q instrument (QIAGEN Sample & Assay Technologies, Germany). For the reactions, tree-step cycling PCR program including 15 min at 95°C initial denaturation and 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C for 40 cycle was performed. Also, a melting curve analysis between 60 and 95°C immediately was performed after PCR to ensure PCR efficiency. All reactions were studied in triplicate.

Data Analysis

Gene expression levels were calculated by $2^{-\Delta Ct}$ ($\Delta Ct = \text{Target gene} - \text{Reference gene}$) method. GAPDH reference gene was used to normalize resulting expression data. GraphPad Prism (Version 6 for Windows) statistical analysis software was used to analyze statistical changes. For the statistical comparisons, Wilcoxon matched-pairs signed rank test was applied during analysis. All statistics were two-tailed and $p < 0.05$ were accepted as statistically significant.

Ethics statement

This present study was reviewed in accordance to the Declaration of Helsinki on medical protocol and ethics. The regional Ethical Review Board of Adiyaman University Ethics Committee approved the study (Approval Number: 2018/1-7).

RESULTS

As a result of the gene expression analysis, we identified significant gene expression variations in odontogenic cysts. In particular, relative gene expression levels of two well-known tumor suppressor genes, RB1 ($p=0,0479$) and TP53 ($p=0.0054$) were found to be significantly decreased in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients (Fig 1).

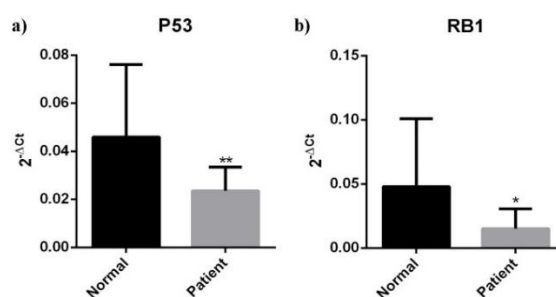


Fig 1. Quantitative expression levels of P53 and RB1 genes in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients.

In addition, gene expression levels of CASP3 ($p=0.0085$) and CASP9 ($p=0.0479$), which are involved in the intrinsic pathway of apoptosis, were found to be significantly reduced in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients (Fig 2). Also, gene expression level of XIAP (0.0353), which is an apoptosis inhibitor gene, was found to be significantly advanced in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients. Moreover, gene expression level of AIFM3 ($p>0.05$), an apoptosis inducing gene, was also found to be diminished but this variation was statistically not significant (Fig 2).

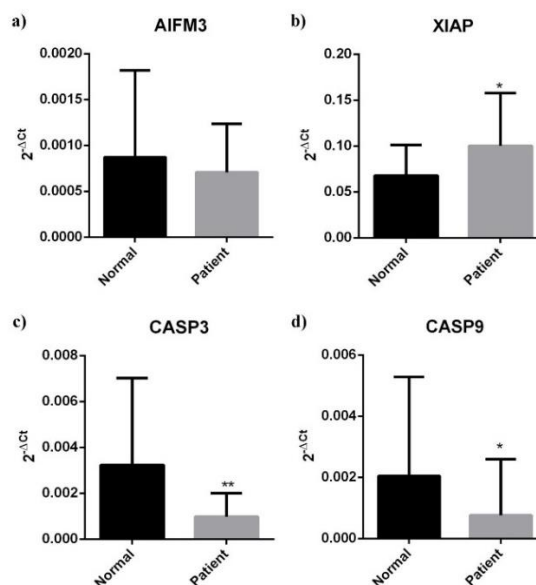


Fig 2. Quantitative expression levels of AIFM3, XIAP, CASP3 and CASP9 genes in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients.

Furthermore, gene expression level of pro-apoptotic BAX gene was found to be increased in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients (Fig 3). Also, BCL2 gene expression levels were found to

be reduced in issues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients (Fig 3). However, changes in the expression levels of BAX and BCL2 were statistically insignificant ($p>0.05$).

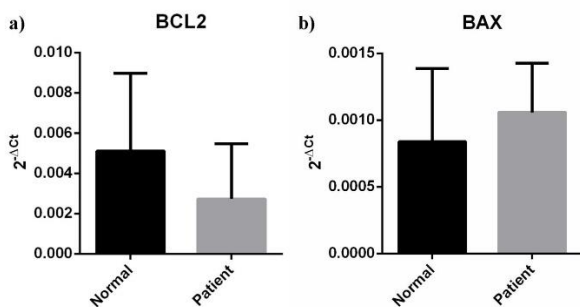


Fig 3. Quantitative expression levels of BCL2 and BAX genes in tissues of odontogenic cysts as compared to adjacent gingival healthy tissue of patients.

DISCUSSION

Increasing mass of evidence indicate that several proteins participated in the formation and progression of tumors are also deregulated in odontogenic cysts. Previous work by Kaczmarzyk *et al.*¹⁴ analysed immunoexpression of PCNA, P53, COX-2, and BCL-2 and reported that these proteins are involved in the physiopathology of odontogenic keratocyst and expression of these proteins were associated with the radiographic evidence of cortical perforation and age of patients. Fatemeh *et al.*¹² also reported significant differential expression of p53 protein in odontogenic cysts. In particular, they established that p53 is highly positive in inflamed odontogenic keratocyst in contrast to non-inflamed odontogenic keratocyst and p53 expression highly decreased in dental follicles.

Furthermore, in a separate study, differential up-regulation of HIF-1 α and CASPASE-3 by determined by immunohistochemistry. Particularly, expression of HIF-1 α was higher in radicular cyst, dentigerous cyst, and ameloblastoma as compared to dental follicle tissues.¹⁷ These findings strongly suggest the contribution of HIF-1 α and CASPASE-3 in the formation of odontogenic cysts and tumors. Moreover, Gadbail *et al.*¹⁵ also demonstrated the role of Ki-67 and p53 in odontogenic keratocyst and ameloblastoma and found that both Ki-67 and p53 expressions are elevated in ameloblastoma as compared to odontogenic keratocyst. Also, both Ki-

67 and p53 expressions were advanced in odontogenic keratocyst than dentigerous cyst, suggesting Ki-67 and p53 protein expression might be useful prognostic biomarker in odontogenic lesions. Sreedhar *et al.*¹⁸ also established the expression status of PCNA and BCL-2 proteins in odontogenic keratocyst and dentigerous cyst. Particularly, they showed that while PCNA was markedly increased, Bcl-2 was significantly markedly in odontogenic keratocyst and dentigerous cyst.¹⁸ In a different study comprising p53, Bcl-2 and Bax proteins, while expression of p53 and Bcl-2 was found to be positively correlated, expression of p53 and Bax protein was found to be negatively correlated.¹⁹

In line with these findings, in tissues of odontogenic cysts, we also identified significant genetic alterations in genes significantly involved in the formation and progression of human cancers. As we all know, loss of tumor suppressive activity is the initial step in formation of tumors and deregulation of these two has been much reported in several types of neoplastic growths and cancers.²⁰ TP53 has been known as “the guardian of genome” because it is activated in harsh conditions, especially in case of DNA damage.²⁰ Once the TP53 is activated, it temporally arrests cell cycle at the G1 phase to allow DNA repair if the DNA damage is reversible. Alternatively, if the damage is irreversible TP53 forces cells to die by apoptosis.²¹ In our study, expression levels of both RB1 and TP53 tumor suppressor genes were found to be significantly reduced in tissues of odontogenic cysts. Our results are also consistent with the previous immunohistochemistry studies. Thus, these findings suggest that these two genes might have important roles in the formation of these cysts and might be reliable biomarkers in characterization of these cysts.

Additionally, deregulation of apoptotic mechanisms is a chief contributor of the development of various types of disease ranging from cancer to neurological disorders and might have significant influence in developing therapeutic strategy.²² Particularly, growing evidence suggests that tumor cells are extremely

resistant to apoptotic cell death which eliminates damaged cells.²² Resistant to apoptosis emerges as a result of differential overexpression of anti-apoptotic genes such as Bcl-2 and IAP (inhibitors of apoptosis proteins) family of proteins or reduced expression of pro-apoptotic members such as Bid and Bax.^{22,23} Similar to tumors, odontogenic cysts can also acquire resistance programmed cell death by deregulation of pro-apoptotic and anti-apoptotic protein members as we mentioned above. Accordingly, we also evaluated the gene expression levels of pro-apoptotic and anti-apoptotic members of the programmed cell death pathway as well as protein involved in the initiation (caspase-9) and execution (caspase-3) phases of apoptosis. As a result, we recognized that while gene expression levels of anti-apoptotic XIAP was significantly increased in odontogenic cysts, expression levels of apoptosis initiator caspase-9 and executioner caspase-3 was found to be significantly decreased in odontogenic cysts as compared to adjacent gingival healthy tissue of patients. Our findings are also consistent with previous findings about caspase-3 protein expression in immunohistochemistry. These findings supports the notion that apoptosis pathway is markedly deregulated in the formation and progression of odontogenic cysts.

Taken together, odontogenic cysts seems to be acquire molecular changes acquired by tumors by downregulation of tumor suppressor and pro-apoptotic proteins and up-regulation of anti-apoptotic proteins. Here, we identified significant differential expression of genes involved in the formation and progression of tumors.

CONCLUSIONS

In conclusion, the findings of the present study indicates that RB1, TP53, XIAP, CASP3 and CASP9 might have significant impact in formation odontogenic cysts and responsible for the increased cell proliferation in these tissues. On the other hand, there are several limitations of our study. The most important one is the small sample size. In the upcoming studies, it is of great interest to determine the role of these genes in larger study populations. Also, comparing the

gene expression results between types of odontogenic cysts is of great interest. Further in vitro studies might also be conducted to demonstrate the functional roles of these genes in the cysts development. Lastly, identifying microRNA and long non-coding RNA regulators of these genes is of great interest to illuminate exact mechanism of pathogenesis.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

Odontojenik Kistlerde Kanser İlişkili Genlerin Düzensiz Ekspresyonu

ÖZ

Amaç: Bu çalışmanın amacı odontojenik kistlerde RB1, TP53, XIAP, BCL2 AIFM3, BAX, CASP3 ve CASP9 genlerinin ayırt edici ekspresyonlarının ortaya konulmasıdır. **Gereç ve Yöntemler:** Bu çalışmaya odontojenik kist tanısı konulan toplam 15 hasta dahil edilmiştir. Gen ekspresyonu analizi için cerrahi operasyon sırasında hastaların kist ve bitişik gingival sağlıklı dokuları toplanmıştır. RB1, TP53, XIAP, BCL2 AIFM3, BAX, CASP3 ve CASP9 genlerinin kantitatif ekspresyon seviyelerinin belirlenmesi amacıyla real-time PCR yöntemi kullanılmıştır. Gen ekspresyon seviyelerinin optimize edilmesi amacıyla GAPDH referans geni kullanıldı. **Bulgular:** Hem RB1 hem de TP53 genlerinin ekspresyonu, sağlıklı dokularla karşılaştırıldığında odontojenik kist dokularında belirgin bir şekilde azalmıştır ($p<0,05$). Ayrıca, odontojenik kist dokularında CASP3 ve CASP9 genlerinde sağlıklı dokularla karşılaştırıldığında istatistiksel olarak anlamlı düşüş saptanmıştır ($p<0,05$). Buna karşılık, XIAP ekspresyonunun anlamlı olarak arttığı saptanmıştır ($p<0,05$). Her ne kadar BCL2, AIFM3 ve BAX genleri de odontojenik kist dokularında ayırıcı bir şekilde eksprese edilmiş olsa da, bu değişikliklerin istatistiksel olarak anlamsız olduğu saptanmıştır ($p>0,05$). **Sonuçlar:** Bu çalışmanın sonuçları, RB1, TP53, XIAP, CASP3 ve CASP9 genlerinin, odontojenik kistlerin oluşumunda önemli rol oynayabileceğini ve bu dokulardaki artan hücre çoğalmasından sorumlu olabileceğini göstermektedir. **Anahtar kelimeler:** Gen ekspresyonu, odontojenik kistler, tümör baskılayıcı genler.

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