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Research Article

ESTIMATION OF WNT BETACATENIN LEVELS IN GENERALIZED CHRONIC AND AGGRESSIVE PERIODONTITIS-A COMPARATIVE CROSS SECTIONAL HISTOCHEMICAL STUDY

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INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by the enhanced expression of inflammatory mediators leading to alveolar bone resorption.[1] Although, oral bacterial infection is a major factor of Periodontitis, its progression and severity depends upon interplay between genetic and environmental factors. . Periodontopathic bacteria produce many virulence factors, such as lipopolysaccharide(LPS) including the production of inflammatory cytokines which leads to the stimulation of host cells, thus playing an important role in mediating and controlling cellular interactions[1].

Binding of various cell adhesion molecules and chemokines initiates signals that regulate leukocyte motility. Various signals are mediated through Toll like receptors (TLR's), which promote signal transduction through its cytoplasmic receptors. Among the various signal transduction pathways like MAPK/ERK pathway, cAMP dependent pathway, IP_3/DAG pathway, etc. Wnt/ß-catenin pathway plays an important role in cell signaling from outside of the cell through surface receptors to the inside of the cell[1].

Wingless, a second chromosome recessive mutation in Drosophila was first reported by Sharma and Chopra in 1975. It was characterised as a segment polarity gene in Drosophila, which is essential for embryonic segmentation and patterning[1]

Wnt proteins are evolutionarily conserved secreted glycoproteins mediating short-range paracrine signalling. The name "Wnt" was coined after the discovery that the Drosophila gene for wingless (wg) and the murine oncogene int-1 are conserved orthologous. Wnt proteins bind with serpentine receptors of the Frizzled (Fzd) family on cell membrane to trigger several distinct signalling cascades: the canonical or Wnt/ß-catenin pathway; the Wnt/Ca+2 pathway involving Protein Kinase A; the planar cell polarity pathway; and a pathway involving Protein Kinase C that functions in muscle myogenesis.[2]

There are 19 human Wnt genes, several of which encode additional, alternatively spliced isoforms. Canonical Wnts (e.g. Wnt1, Wnt3A and Wnt8) stabilize ß-catenin, thereby activating transcription of Tcf/LEF target genes. Noncanonical Wnts (e.g. Wnt4, Wnt5A [1,19,20] and Wnt11) activate other signaling pathways, such as the planar-cell-polarity (PCP)-like pathway that guides cell movements during gastrulation[3]

This research is focused on canonical Wnt/ß-catenin pathway that is mediated by the stabilization of cytoplasmic ß-catenin and its consequent nuclear entry as a transcription activator. A simple outline of the current model of Wnt/ß-catenin pathway is presented in **Figure 1.**

Membrane ß-catenin is a component of intercellular adherent junctions, where it directly interacts with E-cadherin and forms a

dynamic link to the cytoskeleton. Additional catenin's such as γ-catenin and δ-catenin have also been identified.[5]

Emphasizing the functions of Wnt/ß-catenin pathway several investigators have reported a variety of important roles in Embryogenesis[4], severe tooth agenesis or oligodontia[4], bone growth and remodelling[6], , stem cell renewal[8], skeletogenesis[9] chronic inflammatory diseases like rheumatoid arthritis[21], risk factors like smoking on periodontium and human airway epithelium.[18,22] periapical bone lesions[13] oral cancers metastasis[7,23,24].

Human gingival fibroblasts (hGFs), periodontal ligament (PDL) cells and osteoblasts, are major fibroblastic cells in the periodontal tissue. More recent data suggest the importance of Wnt/ß-catenin pathway in periodontal conditions and ß-catenin localisation in periodontal ligament cells [101112 1415161720].

Thus, studying the function of Wnt/ß-catenin is a par powerful approach to delineate the roles that the canonical Wnt signaling pathway may have in a given parts of cell type.

Hence the aim of this research was to localize the expression of ß-catenin in gingival tissues of subjects with healthy periodontium, chronic periodontitis and generalized aggressive periodontitis. This study could add into the ever challenging growth of research in the field of genetics and periodontal disease.

MATERIAL AND METHODS

A total of 90 randomly selected Subjects who visited the outpatient Department of Periodontics, P.M.N.M. Dental College and Hospital at Bagalkot, Karnataka. with the age range from 18 to 60 years, gender matched were included in this study. Gingival tissue samples from the selected subjects were obtained for the ß-catenin expression. Samples were grouped as follows:

Group I

30 subjects with healthy periodontium.(systemically healthy and showing absence of clinical manifestations of periodontal disease)

Group II

30 patients with chronic periodontitis.(patients comprising of at least 20 natural teeth and a minimum of six teeth with periodontal pockets ≥ 5mm and clinical attachment loss ≥3-4 mm)[25]

Group III

30 patients with generalized aggressive periodontitis (patients with proximal attachment loss of ≥5mm affecting at least three teeth other than first molars and incisors)[25]. Were included, where as subjects with history of systemic diseases like diabete mellitus, hepatitis, heart diseases, rheumatoid arthritis and other systemic diseases that can alter the course of periodontal disease, smokers, those who have had antibiotics and analgesics within last six months were excluded from study. A proforma was designed to obtain an insight into the patient's chief complaint, medical and dental history and provided a systematic and methodical recording of the relevant information. Clinical parameters like Gingival index **(Loe and Sillness 1963)** Probing pocket depth (PPD). Clinical attachment loss (CAL) were recorded[26,27]. Under local anaesthesia, rectangular biopsies were obtained by surgical excision from labial/buccal surface of gingiva[28]. The gingival tissue sample was stored and transported in buffered formalin solution for immunohistochemical analysis.

Evaluation of the staining for ß-catenin

Initially the type of staining was evaluated broadly in two categories: - Based on localization of **ß-catenin** stain

1. Membranous
2. Membranous + cytoplasmic

Assessment of ß-catenin positive cells was performed using double headed light microscope at ×10 and ×40.The criteria used to define ß-catenin antigen positive cells were brown staining in cell membrane;the pattern was described as normal when staining was exclusively membranous and of similar intensityto adjacent normal epithelium. Abnormal staining included discontinuous or absent membranous staining, with or without cytoplasmic staining. Presence of immunostaining in the cell membrane of various layers of epithelium was evaluated in randomized six fields/intensity of positively stained cells as percentage expression at 40x and graded as shown in table. The current grading criteria were chosen as defined by **Simionescu et al (2008)**[29].The expression of ß-catenin was interpreted by an experienced pathologist who was blinded as towhich group the patient belonged, to eliminate bias. Interpretation of ß-catenin expression was done and the results were tabulated. (Table 1)

The data obtained was statistically analyzed and the data was described as numbers and percentage. Statistical significance was set at p-value less than <0.05.

RESULTS

Accordingly when the expression of β-catenin was analyzed in the gingival tissue epithelium, appreciable staining was observed. β-catenin expression in the gingival epithelium in terms of degree 1+ was not observed in any of the three

groups. In healthy controls (n=30) β-catenin expression in the gingival epithelium was degree 0 in 76.6% (n=23), degree 2+ in 3.33% (n=1), degree 3+ in 13.33% (n=4), degree 4+ in 6.67% (n=4). Likewise, group 2 CP (n=30) showed degree 0 in 50% (n=15), degree 2+ in 6.67% (n=2), degree 3+ in 13.33% (n=4), degree 4+ in 30% (n=9). Where as in group 3 GAP (n=30), β-catenin expression was degree 0 in 46.67% (n=14), degree 2+ in 3.33% (n=1) and degree 3+ in 3.33% (n=1) degree 4+ in 46.67% (n=14).

The overall expression of β-catenin in gingival tissue epithelium was statistically significant among three groups (CHI SQUARE TEST H=14.0286, p=0.0293*). The expression of β-catenin in gingival tissue epithelium was higher in CP group compared to healthy group which was not statistically significant p<0.05 (CHI SQUARE TEST H=6.4723, p=0.0293). Similarly, the difference in expression of β-catenin in gingival tissue epithelium was significant (P<0.05) when healthy controls were compared with GAP subjects (CHI SQUARE TEST H=12.9893 p=0.0052*). The difference in expression of β-catenin in gingival tissue epithelium was not statistically significant when CP subjects were compared with GAP subjects (CHI SQUARE TEST H=3.2553, p=0.3542)

Spearman's ranks correlation method showed positive correlation between β-catenin expression and clinical parameters like PD and CAL in healthy controls but not statistically significant, while non-significant negative correlation was observed with GI scores. A non-significant negative correlation was observed between β-catenin expressions with GI, PD, CAL scores in chronic periodontitis subjects. Furthermore, GAP group showed a non-significant negative correlation between β-catenin expression and PD and CAL, while a positive correlation was found with GI scores which were not statistically significant (**Graph 1 and Graph 2**).

DISCUSSION

In the oral mucosa, epithelium shows adaptation to different mechanical demands and maintains its structure by a process of continuous cell renewal. However, we now believe that the epithelial cells play an active role in innate host defense by responding to bacteria in an interactive manner. Periodontal epithelial cells may respond to bacteria by increased proliferation, alteration of cell signaling events, changes in differentiation and cell death and ultimately alteration of homeostasis[30]

Nowadays, it is widely accepted that periodontitis is not caused by single or a limited group of pathogens, but rather caused by polymicrobial synergy and dysbiosis [31]. Chronic periodontitis is the most prevalent form of periodontitis. Whereas, Aggressive periodontitis is a rare disease of the periodontium occurring in an otherwise healthy adolescent which is characterized by a rapid loss of alveolar bone involving about more than one tooth of the permanent dentition.

Bone remodeling requires a complex network of systemic hormones and local factors for osteoprogenitor lineage cells to progress through stages of differentiation. Constituents of the Wnt/β- catenin pathway are among these factors; this pathway increases bone mass through mechanisms including renewal of stem cells, stimulation of pre-osteoblast replication, in duction of osteoblastogenesis, and inhibition of osteoblast apoptosis[13].

ß-catenin is a widely expressed 90-kDa protein with dual functions in cell adhesion and Wnt signaling .At the membrane, ß-catenin forms complexes with E-cadherin to generate complexes responsible for maintaining the structural integrity of many epithelial tissues.

In simple terms, the presence of ß-catenin in the cytoplasm/nucleus can be viewed as evidence of Wnt signaling activity in a tissue. In tissue sections this is most commonly visualized by immunohistochemistry using antibodies recognizing ß-catenin.

The working hypothesis for the currentcross sectional study was to assess the expression of ß-catenin in gingival tissues in the context of various types of periodontitis viz chronic periodontitis and aggressive periodontitis. We also intended to correlate the levels of ß-catenin with the clinical parameters among the three groups so as to obtain an insight into the probable in pathogenesis of periodontal diseases.

In this experimental study,study population included both males (44%) and females (57%). healthy (25.67±8.06 years) and GAP subjects (31.0±5.65years) were much younger as compared to CP group (42.67±9.16 years). According to **Barker and Brown (2008)**[32] levels of ß-catenin present in adhesion complexes at the membrane are generally not influenced by Wnt signaling. This is in stark contrast to ß-catenin levels in the cytoplasm and nucleus, which vary dramatically according to the status of the Wnt signaling pathway. Findings in our study followed the same protocol, keeping in mind the type of staining as to whether it is membranous or membranous + cytoplasmic, the healthy group (n=30) showed 73.33%(n=22) membranous, 23.33% (n=7) membranous + cytoplasmic, while (n=1) sample (3.33%) did not take up any stain. The CP group (n=30) demonstrated 46.67 % (n=14)

membranous, 50% (n=15) as membranous + cytoplasmic, whereas (n=1) sample (3.33%) did not take up any stain. Expression of ß-catenin in gingival epithelium of GAP group(n=30) in terms of type of staining was found to be comparatively higher than the other groups,viz 36.67% (n=11) membranous, 53.33% (n=16) membranous + cytoplasmic) and (n=3) samples 10% did not elucidate staining characteristic, and when evaluated carefully the staining pattern in GAP group was found to be involving all the layers of epithelium as well as cytoplasm , the values between the three groups were found to be significant where as they were statistically non-significant on pair wise comparison..

Since we were able to differentiate precisely the type of staining and more than 90% of samples being tested positive with good intensity of staining, propelled us to make our study more specific. Interestingly immunohistochemical staining demonstrated that ß-catenin was expressed in epithelial layer of gingival tissues in all the three groups.

Our data showed an altered expression of ß-catenin; the differences in staining pattern between healthy epithelium and CP and GAP in our series supported the existence of a definite relationship between the alterated expression of ß-catenin and the inflammatory process. High cytoplasmic expression of catenin's is probably due to an increase of the free ß-catenin fraction in inflammatory cells.[4]

Previous reports demonstrated that ß-catenin serves as an effector of mechanical signals in PDL cells.[12] There was an increase in the amount of staining for dephosphorylated ß-catenin in the nucleus of cells. The absence of any increase in dephosphorylated ß-catenin in the cytoplasm in their research suggested its rapid translocation to the nucleus, which is in contrast to current study that showed ß-catenin in cytoplasm of CP and GAP samples.

In the early researches**, Na liu et al (2011)**[16] has shown the higher levels of ß-catenin in periodontal stem cells of periodontitis subjects than in periodontal stem cells obtained from a healthy microenvironment. Though the results of the study showed higher ß-catenin levels in periodontitis, clear emphasis was not given as to which type of periodontitis was under consideration. Our study is the first of its kind to evaluate the expression of ß-catenin in gingival epithelium of chronic periodontitis and generalized aggressive periodontitis.

Overall expression of ß-catenin in gingival epithelial tissues was significantly higher in CP and GAP participants compared to healthy subjects. There is no direct evidence supporting the over-expression of ß-catenin in gingival tissues of aggressive periodontitis patients as there are no other published studies affirming its effects in aggressive periodontal disease sites. The current study also showed over all positive correlation with periodontal parameters like (GI, PPD, CAL), with significant P value equal to 0.0252 (P<0.05) and 0.0230 (P<0.05) with PPD and CAL respectively. However GI scores were found to be insignificant suggesting that ß-catenin is expressed higher in periodontitis involving more damage to deeper structures of periodontium.

Owing to the aforementioned findings of the studies done **by Donald A glass et al (2005) Suda T et (2009) Jung Sun Heo and Seung Youp Lee (2010) Sundaralingam Premaraj et al (2011),** it can be concomitantly said that expression of Wnt/ß-catenin via Wnt pathway is essential for OPG production to stimulate osteoblast formation , one thing can be postulated from findings of our study that the higher expression of ß-catenin in GAP and CP as compared to healthy controls, might be pertaining to compensate the rapid amount of bone loss in these diseases[09,10,11,12,25].

The present study was mainly focused on canonical Wnt/ß-catenin pathway; in periodontitis canonical pathway is known to regulate bone homoestasis[4]. Activation of canonical pathway leads to more bone formation; on the contrary inhibition of this pathway leads to bone destruction.[33]on the basis of above findings we can speculate that the expression of ß-catenin in CP and GAP is must to combat the lethal bone damage that occurs in period of quiescence and remission in these diseases.

Moreover, these findings have important implications for the development of immunomodulatory interventions and strategies for fine-tuning the host response to minimize the destructive aspects of chronic and aggressive periodontitis and maximize the protective aspects of the periodontal host response.

Future therapeutic interventions should be carried out for easy and effective expression of ß-catenin in inflammatory microenvironments and its clinical implications on periodontal health in detail.

CONCLUSION

We anticipated these findings, previous data suggested that the expression of ß-catenin is increased in periodontitis; we have shown for the first time the expression of ß-catenin in Aggressive periodontitis. Moreover the expression of ß-catenin by human gingival epithelial cells in aggressive periodontitis subjects was higher than chronic periodontitis

and healthy subjects. Two possible explanations can be drawn out from our immunohistochemical study, the first one is formally being the localization of cell membranous or cell cytoplasmic ß-catenin in gingival epithelial cells, and secondly the extent of ß-catenin expression in the layers of epithelium as well as percentage positivity in cytoplasm.

Integrated by the evidence of role of Wnt/ ß-catenin pathway in bone physiology, in inflammation, Wnt/ß-catenin appears to be an important target for intervention in periodontitis, and in this context we postulate that Wnt/ß-catenin signaling is must and should always be active and functioning in diseases like chronic and aggressive periodontitis, because it helps in bone formation.

This notion could be tested in future studies in preclinical models, hopefully paving the way for clinical trials. ß-catenin might be applicable therapeutic targets for preventing alveolar bone resorption in periodontitis.

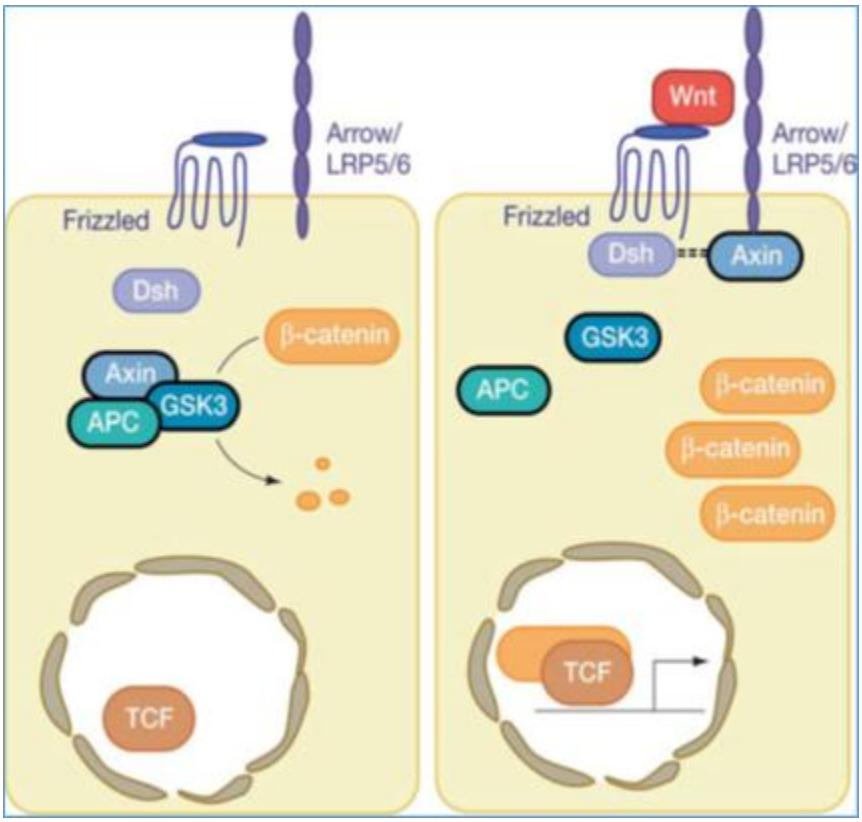
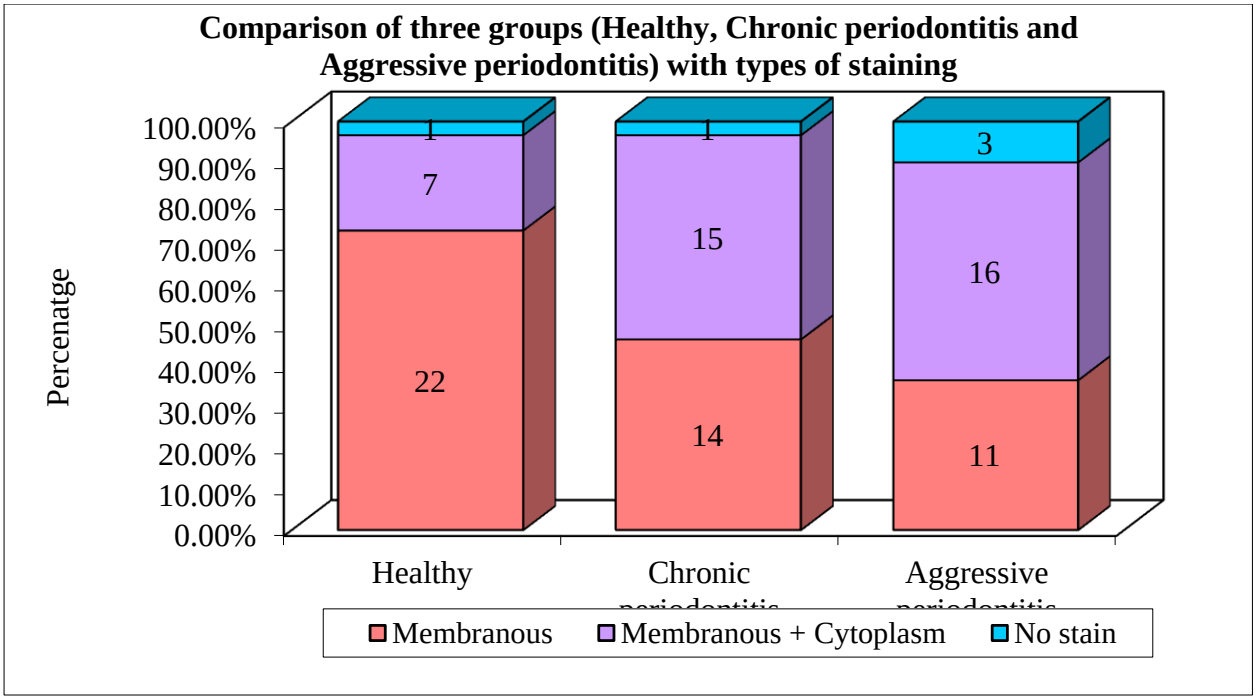


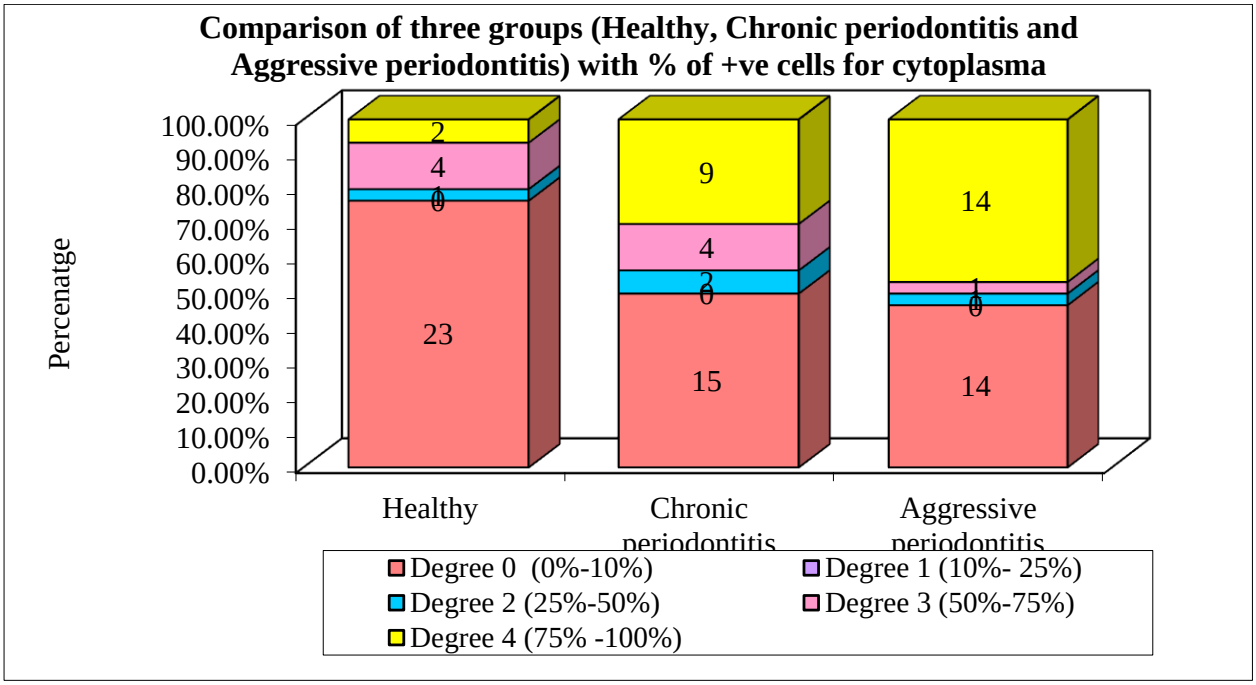
Fig. 1: The canonical Wnt signalling pathway. In cells not exposed to a Wnt signal (left panel), β-catenin is degraded through interactions with Axin, APC, and the protein kinase GSK-3. Wnt proteins (right panel) bind to the Frizzled/LRP receptor complex at the cell surface. These receptors transduce a signal to Dishevelled (Dsh) and to Axin, which may directly interact (dashed lines). As a consequence, the degradation of β-catenin is inhibited, and this protein accumulates in the cytoplasm and nucleus. β-catenin then interacts with TCF to control transcription. Negative regulators are outlined in black. Positively acting components are outlined in color

Table 1:

Grading criteria	Percentage of positive cells in cytoplasm
0 degree	under 10% positive cells
1 degree	between 10–25% positive cells
2 degrees	between 25–50% positive cells
3 degrees	between 50–75% positive cells
4 degrees	over 75% positive cells



Graph. 1:



Graph. 2:

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