Cell cycle inhibitors in oral leukoplakia: A review

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Abstract:

Cell cycle is a complex and coordinated process characterized by orderly progression in the life cycle of cells orchestrated by cyclins and cyclin dependent kinases (CDK) and their inhibitors. The kinases drive the cell cycle by phosphorylating critical target proteins that are required for progression of cell to the next phase of cell cycle. This progress is tightly regulated by a group of cell cycle inhibitors known as CDK inhibitors which may be responsible for maintenance of cell cycle and proliferation arrest once the cells' developmental fate has been reached. Cell cycle inhibitors may act as checkpoints to identify the defective components of the cell thereby aiding in its repair and hence inactivation of these genes may interfere with the terminal differentiation leading to unrestricted proliferation and tumorigenesis. Cell proliferation is an important property of malignant tumor cells and dysregulation of genes governing the cell cycle is of considerable significance in the development of oral squamous cell carcinoma. Most OSCC generally develop by the malignant transformation of potentially malignant disorders the most common of which is leukoplakia. Hence evaluation of these genetic alterations in leukoplakia can be useful in assessment of the potential for malignant transformation and may also aid in attempting any therapeutic strategies at this stage. This review aims to provide data related to the role of cell cycle inhibitors in oral leukoplakia highlighting their role in malignant transformation.

1. Introduction

Cell division consists of two consecutive processes mainly consisting of DNA replication and segregation of daughter chromosomes into two separate cells. Replication of DNA occurs in a specific part of the interphase known as S phase (Vermeulen et al 2003). The transition from one cell cycle phase to another occurs in an orderly fashion and is regulated by cellular proteins. The molecular principles of cell cycle regulation have been defined largely in yeast, and are applicable in human beings which points to a number of surveillance check points that monitor the cell cycle and halt its progression (Cheng 2004). In mammalian cells, the cell cycle machinery which regulates the cell cycle operates mainly in the G1 phase (Cheng 2004). The key regulatory proteins of the cell cycle include the cyclin-dependent kinases (CDK's) which belong to the family of serine/threonine protein kinases that are activated at specific points of cell cycle (Vermeulen et al 2003). The CDK's join the regulatory proteins called cyclins to drive the cell through the complete cycle (Dickson & Schwartz 2009). The pattern of cyclin expression defines the cell progression and there are at least nine CDK's (CDK 1-9) and many cyclins which interact upon activation by specific phosphorylation (Kaldis et al 1998). While CDK's are positively regulated by cyclins through phosphorylation mechanism, the cyclin-CDK complex are also negatively regulated by the CDK inhibitors.

CDK inhibitors also referred to as CKI's act as important checkpoints responsible for the maintenance of cell cycle and proliferation arrest once the cells' development fate has reached. The CDK inhibitors either bind to CDK alone or to the CDK-cyclin complex and thus regulate CDK activity. There are two main classes of CKI's namely the Cip/Kip family which includes p21, p27 and p57 as its components and INK4 family includes p16, p14 and p20. The function of Cip/Kip family is mainly to inactivate cyclin D/CDK bond while INK4 family functions by blocking the cell cycle. The disruption of these check points is one of

the mechanism by which the tumor cell exhibits abnormal proliferation.

Oral leukoplakia is the most frequently encountered potentially malignant disorder of the oral mucosa with an increased potential for malignant transformation. The histopathological description of oral leukoplakia is generally described as epithelial dysplasia based on the architectural and cytological changes observed in the biopsy tissue (Warnakulasuriya et al 2008). While leukoplakia generally has a malignant transformation rate of less than 2%, those with histological evidence of epithelial dysplasia are reported to undergo malignant transformation in the range of 1.1% to 17.5% (Napier & Speight 2008). While it is generally accepted that the malignant transformation rate increases with severe grade of epithelial dysplasia, no clear consensus can be drawn regarding the behavior of non-dysplastic lesions. In addition to clinical and histological changes, these lesions also show widespread genetic and molecular alterations. Molecular assessment of these lesions may thus help in early diagnosis and prevention of malignant transformation.

This paper mainly reviews the role of cell cycle inhibitors in oral leukoplakia and its potential for malignant transformation.

2. Cell cycle and its inhibitors

Cell division consists of two consecutive processes: mitosis, which is the process of nuclear division and; interphase which is the period between two mitoses. The interphase is characterized by G1, S and G2 phase. DNA replication occurs in S phase. The gap phase prior to the DNA replication is G1 phase where the cell is at preparatory stage and G2 phase occurs after DNA synthesis where the cell prepares for mitosis. Cells in the G0 phase includes non-growing, non-proliferating cells in the human body (Kumar et al 2015).

The cyclin dependent kinases (CDK's) regulate the cell cycle in the presence of cyclins. Expression of cyclins is cell cycle phase- dependent and is regulated transcriptionally, post-transcriptionally as well as translationally/

post-translationally (Lundberg & Weinberg 1999). Cyclins D1, D2 and D3 bind to CDK 4 and 6 in order to gain entry in G1 phase (Sherr 1994). Cyclin E binds to CDK2 to regulate progression from G1 to S phase (Ohtsubo et al 1995). Cyclin A interacts with CDK2 and this is required during G2 phase. For entry into M phase cyclin A interacts with CDK1 (Arellano & Moreno 1997). In somatic cells, movement through G1 and S phase is driven by the active form of cyclin D/CDK4,6 complex and subsequent phosphorylation of RB protein (Classon & Harlow 2002). The retinoblastoma (Rb) protein is a key switch at R point and phosphorylation results in structural conformation of proteins that initiate or inhibit cell physiological events (Todd et al 2002). Once Rb is phosphorylated, it partially releases critical transcription factor E2F-1 that turns on a series of genes coding for cyclin A and E that forms a complex with CDK2. The complex leads to complete release of E2F followed by the transcription of series of genes responsible for S-phase progression and DNA synthesis (Bartek & Lukas 2001).

Cyclin D1 is a key component of cell cycle progression and interacts closely with the Rb protein. In the hypo-phosphorylated state, Rb protein acts as a tumor suppressor and contributes to cell cycle regulation at the G1 to S checkpoint by suppressing gene transcription that is required for entry into the S phase. The cell cycle is then arrested in G1. In response to mitogenic signals, CDK4 and CDK6 form a complex with their regulatory subunit, cyclin D1, which phosphorylates the Rb protein, reducing its ability to suppress gene transcription. Controlled phosphorylation and deactivation of the Rb protein by the CDK4/6 complex is essential to progression of the normal cell cycle (Shepperd & MCarthur 2013). In malignant cells, unrestricted CDK4/6 pathway activity can result from alterations in the expression of cyclin-dependent kinases and their regulatory mechanisms. This unhindered cell cycle stimulation yields a growth advantage and

uncontrolled cell proliferation (Shapiro 2006).

Cell cycle regulation is crucial in tumorigenesis and depends on the activities of CDK's which are positively regulated by mitogenic growth factors and negatively by CDK inhibitors (CKIs). These CKI's may either bind alone to CDK or to the CDK-cyclin complex, thereby regulating their activity. There are two main classes of CKIs based on their origin, structures and CDK specificities. They are the Cip/Kip family and INK4 family (Sherr and Roberts 1995). The Cip/Kip family encodes for p21, p27 and p57 which binds to both cyclin and CDK subunits thereby modulating the activities of cyclin D-, E-, A-, and B-CDK complexes. The INK4 gene family encodes p16, p15, p14 and p18 all of which bind to CDK 4 and 6 thus inhibiting their kinase activity by interfering with their association with cyclin D (Sherr and Roberts 1999). While the proteins of both the family share a conserved N-terminal domain, they diverge in the remainder of their sequence, suggesting that each of these proteins could have distinct functions and regulation (Besson et al 2008).

2.1. Cyclin dependent kinases inhibitors

The activity of CDK's in cell cycle regulations is counteracted by their inhibitors known as cyclin dependent kinase inhibitors (CKIs) which function by either binding to CDK alone or to the CDK-cyclin complex. As already described, CKIs belong to two broad family of proteins namely Cip/Kip proteins and the INK4/ARF family.

The Cip/Kip proteins are intrinsically unstructured and they adopt specific tertiary conformations only after binding to other proteins (Lacy et al 2004). The member of this family namely p21, p27 and p57 function by inactivation of G1 cyclin- CDK complex and cyclin B-CDK1 complex. In addition, they can also modulate cell cycle progression via inhibition of components of the replication machinery. An important protein of this group, p21 was first reported to bind to proliferating cell nuclear antigen (PCNA) via its C-

terminus thereby blocking progressive DNA synthesis (Besson et al 2008). This protein is a critical downstream target in the p53-specific pathway of growth control, and can also be induced by p53 independent pathways in relation to terminal differentiation. The p21 gene which encodes this protein is present on chromosome 6p21.2 regions. In normal cells this protein predominantly exists in quaternary complexes with cyclins, CDKs and PCNA to inhibit the CDK activity and controls the G1 to S transition (Xiong et al 1993). The function of p21 includes differentiation of normal and transformed cells, association with terminal differentiation, senescence and apoptosis through p53 independent mechanisms (Agarwal et al 2008). Another member of this group, p27 was first identified as a CKI due to its ability to block the activity of cyclin E/CDK2 and cyclin A/CDK2 complex in cells arrested in G1 phase by transforming growth factor beta, lovastatin and contact inhibition. The expression of p27 is mainly regulated by ubiquitin-dependent proteolysis (Kudo et al 2005). They act mainly in G0 and early G1 with the primary target being E-type cyclin/cdk2 complexes. Mitogenic growth factor signaling causes loss of p27 and their levels increase in response to differentiation signals (Slignerland et al 2000). By immunohistochemical staining, cells in the prickle cell and granular cell layers show strongly positive staining for p27 in their nuclei, but cells in the basal layer do not. Therefore, p27 is suggested to play an important role in cell cycle arrest in oral epithelial cells (Kudo et al 2005). The degree of p27 expression in various human malignancies is reported to be inversely correlated to malignant transformation of lesions (Kovesi and Szende 2006).

The INK4/ARF family are known to cause inactivation of G1 CDK (4 & 6) and the protein of this group are p15, p16, p18 and p14. The most important member is the p16 which is known to control cell cycle by inhibiting the ability of cyclin D-CDK4/6 complex to phosphorylate retinoblastoma protein (pRb). Thus, inactivation of pRb by phosphorylation causes p16 expression while hypophosphorylated active pRb can

repress p16 expression (Li et al 1994). Inactivation of p16 was found in many cancers including head and neck squamous cell carcinoma and may occur by various mechanism such as point mutations, homozygous deletion and DNA methylation (Papadimitrakopoulou et al 1997)

3. Oral leukoplakia and its malignant transformation

Oral squamous cell carcinoma (OSCC) initiates in a multistep process in which normal cells are transformed into a pre-neoplastic cell followed by its malignant transformation. The majority of oral cancers are preceded by visible mucosal changes that have the propensity to develop into malignancy (Villa and Gohel 2014). These changes are currently grouped together and referred to as potentially malignant disorders (Van der Waal 2014). The concept of denoting these disorders as pre-cancerous is based on the fact that (i) they may undergo malignant changes over a period of time, (ii) they co-exist at the margins of clinically diagnosed carcinoma, (iii) they may share morphological and cytological changes observed in malignant lesions, but without frank invasion and (iv) they may demonstrate chromosomal, genomic and molecular alterations as observed in oral carcinoma (Warnakulasuriya et al 2007). The most common form of potentially malignant disorder is leukoplakia which is defined as a white plaque of questionable risk having excluded other known diseases or disorders that carry no risk of cancer (Warnakulasuriya et al 2007). The process of diagnosing leukoplakia begins with a provisional diagnosis which is made at the initial clinical examination when the lesion is not clearly diagnosed as any other entity. This is followed by a definite diagnosis which is made when any etiological cause other than tobacco/areca nut has been excluded and histopathology has not confirmed any other disorder (van der Wall and Axell 2002).

While leukoplakia is a clinical term, the histopathology of these lesions serve two important functions; to exclude any other definable lesion and to establish the degree of epithelial dysplasia if any (Van

der Waal et al 1997). The term epithelial dysplasia is applied when the tissue demonstrates architectural disturbances along with cytological atypia (Barnes et al 2005), and its presence serves as an important prognostic predictor of malignant transformation. The microscopic appearance of oral leukoplakia varies from a simple hyperplasia characterized by acanthosis without cellular atypia to dysplasia. The histopathological features of epithelial dysplasia includes loss of polarity of the basal cells; presence of more than one layer having a basaloid appearance; drop-shaped rete-ridges; increased nuclear-cytoplasmic ratio; nuclear hyperchromatism; enlarged nucleoli; increased number of mitotic figures; abnormal mitosis; presence of mitotic figures in the superficial half of the epithelium; cellular and nuclear pleomorphism; irregular epithelial stratification; loss of intercellular adherence; keratinization of single cells or cell groups in the prickle cell layer. The dysplastic features can be graded as mild, moderate and severe based on the prominence of the features and may vary depending on the site of the lesion (Warnakulasuriya 2001).

The possible reason for the increase malignant transformation with increase in the severity of dysplasia could be attributed to the accumulation of genomic and molecular alterations that may initiate cancer development. This, however, does not rule out the development of carcinoma from less severe dysplasia. Till date, there is no significant marker to understand and predict the potential of a lesion to undergo malignant transformation. Genetic mutation leading to potentially malignant lesions and oral malignancy mainly affects two set of genes namely the proto-oncogenes and tumor suppressor genes. The progress of a cell through various stages of cell cycle is tightly regulated by various mechanisms and disruption of these regulators may result in unrestrained proliferation of tumor cells. The CKIs are an important regulator of this complex process which undergo alteration thus leading to cell cycle deregulation. The identification and characterization of these cell cycle inhibitors could serve as an

important therapeutic target to prevent the malignant transformation of oral leukoplakia and thus provide a better prognosis.

4. CKIs in oral leukoplakia

Over the years, scientific data has been amassed to assess the role of CKIs in oral leukoplakia and OSCC. The studies pertaining to the role of various CKIs in oral leukoplakia and its malignant transformation are highlighted in the table.

Sl. No	**Author and year**	**Type of CKIs studied and the lesion**	**Results & interpretation**	**Conclusion**
1	Kovesi and Szende (2006)	p27 in oral leukoplakia (homogenous, nodular) and erythroleukoplakia	-Decreased expression in erythroplakia than in other forms of leukoplakia which could be of prognostic value -Increased expression in nodular leukoplakia than homogenous leukoplakia which could be due to defense mechanism against malignant	These gene products could be useful tool for a precise prognosis of oral leukoplakia and its malignant transformation

			transformation	
2	Shintani S et al (2002)	p16, p12 and p27 in normal, oral epithelial dysplasia and OSCC	- Decreased expression of cell cycle inhibitors in dysplasia and OSCC than in normal controls. -Significant loss of expression in OSCC than in epithelial dysplasia	Loss of CKI expression may be associated with poor prognosis and contribute to multistep nature of oral carcinogenesis
3	Visioli et al (2012)	p21 expression between non-dysplastic leukoplakia and dysplastic leukoplakia	Overexpression of p21 in both dysplastic and non-dysplastic leukoplakia than in normal controls was evident. However similar expression profile was seen in both forms of leukoplakia.	Increased expression could represent an attempt to control cell proliferation, which is possibly overcome by other factors which stimulate carcinogenesis and overload the inhibitory function of p21
4	Papadimitrakopoulou	p16 expression in oral	47% of patients	The finding

	et al 1997)	leukoplakia	with oral premalignant lesions demonstrated lack of p16 expression. 8 of these patients developed carcinoma and among them, 5 patients showed complete loss of p16 expression	supports the important role of p16 gene in head and neck tumorigenesis
5	Agarwal et al (1998)	p21 expression in normal, oral leukoplakia and OSCC patients with clinical correlation	The result showed negligible staining in normal controls, 60% of leukoplakia and 68% of OSCC showed positive expression of p21. Expression decreased with increased grades of OSCC.	Heterogeneity in p21 expression was observed in oral SCCs and in premalignant lesions, suggesting that alterations in p21 expression are an early event in oral oncogenesis.
6	Kresty et al (2008)	p16 and p14 expression in proliferative verrucous leukoplakia	Loss of p16 and p14 expression	May contribute to the aggressiveness and high rates

				of malignant transformation.
7	Buajeeb et al (2009)	p16 expression in oral leukoplakia with and without dysplasia and OSCC	No significant difference in p16 expression among the study groups.	p16 could not be used as a reliable marker for oral mucosal dysplasia and malignant transformation
8	Bradley et al (2006)	p16 expression in oral epithelial dysplasia	Loss of p16 expression in 36% of non-dysplasia, 39% mild dysplasia and 66% of moderate/severe dysplasia	Decreased expression of p16 in dysplastic lesions, may reflect the biologic events involving loss of p16 gene function in the pathogenesis of oral cancer. The study also indicates that p16 is not useful in differentiating dysplastic and non-dysplastic oral lesions

				though decreased p16 expression with increasing severity of dysplasia was observed.
9	Nasser et al (2011)	p16, p53, cyclin D1 expression in non-dysplastic leukoplakia, dysplastic leukoplakia and OSCC for comparison	Loss of p16 expression was seen in 32.4% of leukoplakia without dysplasia. The frequency of loss increased linearly with the histopathological state	Loss of p16 is an early and common event in oral carcinogenesis. A combined alteration of various proteins could serve as a biomarker to define high risk leukoplakia patients. Lesions that do not show these alterations can be considered harmless.
10	Ramasubramanian A (2013)	p27, p63 and cyclin D1 in varying grades of epithelial dysplasia	Significantly increased expression of cyclin D1 and p63	The findings may serve as a prognostic marker for any

			with moderately significant decrease in p27 expression with increasing grades of dysplasia was observed.	preceding malignant transformation and in oral cancer progression
11	Tsuzuki et al (2003)	p27, cyclin D1, PCNA expression in oral leukoplakia and apoptotic index.	p27 was expressed in 34.8% of leukoplakia with hyperplasia, 55.4% in leukoplakia with dysplasia and 31.1% in SCC (43.3% in the early stage of SCC, 25.9% in the advanced stage of SCC). The level of p27 expression showed a peak in dysplasia and decreased in SCC	The study suggests that the abundance of p27 in oral leukoplakia may inhibit cell proliferation and lead premalignant tumor cells to apoptosis, and thus is concerned with prevention of tumor progression.

From the data available it is evident that there are contradictory results obtained pertaining to the exact role of CKIs. This could be attributed to inadequate sample size, lack of standardization with respect to the staining pattern, and possible variation in the clinical presentation of the lesion that may influence sample selection. The genetic changes evident in oral

carcinogenesis are complex and there is a dynamic interplay of these changes which might influence the overall pathogenesis of oral cancer and hence it is difficult to attribute the malignant transformation to a single factor. However the following interpretations can be derived from the available data in the literature: a) loss of cell cycle inhibitor proteins are associated with poor prognosis and malignant transformation of oral leukoplakia b) it is necessary to analyze a panel of CKIs rather than an isolated protein to derive appropriate conclusions; and these studies invite exciting opportunities to gain insight into the genetic events that drive the carcinogenesis process and the effects of chemopreventive interventions on possible modification of the changes that define the malignant phenotype.

5. Conclusion

Cell cycle and its inhibitors play a significant role in the malignant transformation of potentially malignant disorders and oral carcinogenesis. But modulation of cell cycle has also been known to contribute to chemotherapy resistance. It is thus prudent to understand the role of CKIs to predict the biological behavior of a suspicious lesion as well as to implement relevant therapeutic modalities. A wide range of anti-cancer drugs directed at CKIs are at different stages of clinical trials and it is only a matter of time before they are available for clinical use.

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