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Targeting of chemoprevention to high-risk potentially malignant oral lesions: Challenges and opportunities

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Abstract

Worldwide, oral cancer is responsible for 170,000 deaths per year. Intervention to prevent this disease is a long sought after goal. Chemoprevention studies have focused on clinicopathological features of potentially malignant lesions (PML) in an effort to prevent their progression to cancer. However, prediction of future behavior for such lesions is difficult and remains a major challenge to such intervention.

Different approaches to this problem have been tested in the past 20 years. Early genetic progression models identified critical regions of allelic imbalance at 3p and 9p, and provided the basis for molecular markers to identify progressing PMLs. Subsequently, technological advances, such as genome-wide high-throughput array platforms, computer imaging, visualization technology and next generation sequencing, have broadened the scope for marker development and have the potential of further improving our ability to identify high-risk lesions in the near future either alone or in combination.

In this article, we examine the milestones in the development of markers for PML progression. We emphasize the critical importance of networks among scientists, health professionals and community to facilitate the validation and application of putative markers into clinical practice. With a growing number of new agents to validate, it is necessary to coordinate the design and

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CONFLICTS OF INTEREST

None declared.

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implementation of strategies for patient recruitment, integration of marker assessment, and the final translation of such approaches into clinical use.

Keywords

Loss-of-heterozygosity; oral potentially malignant lesions; oral premalignant lesions; progression markers; knowledge translation

INTRODUCTION

Oral cancer is a significant global health concern. It is responsible for over 300,000 new cases per year ¹. Most cases are diagnosed at a late stage (from 60 to 80% in high and low resource countries, respectively), resulting in poor 5-year survival rates (30–60%)^{2–4}. Hence, the need is high for earlier detection of the disease and the development of new management strategies. Intervention through chemoprevention is one such possible approach, defined as the use of a potentially wide range of agents to block or reverse carcinogenesis in individuals exposed to risk factor(s) and/or diagnosed with a precursor lesion ⁵. The oral cavity is particularly amenable to such intervention, since it is directly exposed to carcinogenic agents and precursor lesions are easily accessed for clinical evaluation, biopsy acquisition and follow-up.

Chemoprevention studies in the oral cavity began in the early 1980s with the benchmark randomized trials of retinoids by Hong, Lippman and colleagues and continue to this day with trials that use a diverse array of agents ^{6–9}. Together these studies have contributed significantly to the development of evidence-based strategies for chemoprevention by guiding study design and establishing a framework for sample collection and analysis, at the same time providing samples for biomarker discovery. However, major barriers to chemoprevention studies at this site remain including challenges related to identification of oral lesions at high risk for malignant transformation and the difficulty of patient recruitment.

In this article, some of the current efforts to optimize the selection of patients suitable to chemoprevention trials will be discussed. We revisit strategies used to identify patients with lesions at higher risk of malignant progression, exemplified by one of the most promising available methodologies: loss of heterozygosity (LOH) analysis. Finally, alternate approaches that build on dysplasia to identify those at greatest risk to progress are introduced with an emphasis on their potential value alone and in combination to better predict outcome and guide choice of patients for study.

CURRENT LIMITATIONS

As mentioned, one of the major obstacles in chemoprevention studies to date has been the inability to identify lesions with a high risk of malignant transformation. Clinical lesions in the oral cavity are very heterogeneous, varying in color, size, texture and anatomic site, with the percentage that progress to malignancy showing a wide range in different studies (1.1–25 %) ^{10–12}. Currently, definition of risk for these lesions relies on their biopsy and

histological assessment for dysplasia by most clinicians, although specialists may also combine other features such as clinical appearance, size and toluidine blue staining results of PMD. Most trials use a combination of clinical change and histology to identify patients for study and to monitor impact of drugs. The degree of heterogeneity of oral dysplasia is further affirmed in a meta-analysis by Mehanna et. al.¹³. Malignant transformation rates varied widely across studies (0%–36.4%), independent of the follow-up time. Moreover, the time interval to malignant transformation for dysplastic lesions ranged from 0.5 to 16 years, with no significant differences of this rate in subgroup analysis by dysplasia grade and treatment modality.

Using histology as a “gold standard” for predicting progression has major limitations. Severe grades of dysplasia are generally considered to have higher transformation rates^{14–16}; however, not all such lesions will progress. Equally important, patients with high-grade lesions are difficult to accrue in large numbers for study due to the difficulty in accessing such cases in the community. Thus, the majority of cases that have been involved in chemoprevention trials show minimal to no dysplasia (i.e. hyperplasia, mild or moderate dysplasia). Histology alone is a poor predictor of which low-grade lesions will undergo malignant change: some dysplastic lesions disappear over time and others remain unchanged, while a few transform to cancer¹⁷. Moreover, hyperplastic and normal appearing mucosa have also been reported to undergo malignant transformation^{18–20}. Further complicating this issue is the fact that histological grading is largely subjective, resulting in significant inter- and intra-observer variability²¹.

Given the aforementioned difficulties and the utilization of primarily low-risk lesions, it is perhaps not surprising that randomized trials to date have had low event rates in control arms with only a few subjects likely to benefit from effective agents. It has long been known that better identification of high-risk lesions would result in more effective trials, where toxicity is more justified since risk is high, and where targeting of lesions could reduce sample size and yield outcome data quicker. In addition, identification of low-risk lesions would spare such individuals from unwarranted intervention. However, moving towards this more fruitful framework has been difficult.

LOSS OF HETEROZYGOSITY AS PROGRESSION MARKER: HISTORY AND LESSONS LEARNED

A genetic progression model for oral cancer

The development and validation of predictive markers is a long-term process, from initial discovery to retrospective and then prospective analysis. This is exemplified in one of the earliest approaches that have been developed, which uses loss of heterozygosity (LOH) analysis of key chromosomal regions to predict risk. This well-known PCR-based assay has been shown to predict malignant progression of oral lesions in retrospective studies and is the first and only assay to date that has been prospectively validated for such ability in an independent cohort of patients from community settings. In addition, two recently completed trials have used this approach to select patients for accrual (Erlotinib: ClinicalTrials.gov identifier: NCT00402779 and cetuximab: ClinicalTrials.gov identifier: NCT00524017).

Some of the key steps used to develop this assay are described below along with an indication of strategies used to build patient accrual for such studies.

Interest in the use of alterations in DNA copy-number as revealed by LOH at select chromosome sites was initially triggered by a hallmark publication in 1996 from Sidransky and Califano at Johns Hopkins²². The study used as its basis a set of microsatellite markers for specific chromosome loci that had strongly associated with invasive cancers, testing them on DNA from microdissected, archival tissue with and without dysplasia. This paper was the first to delineate a group of molecular changes that associated with early premalignant stages of head and neck squamous cell carcinoma (HNSCC) cancer, establishing the basis for using early LOH events, such as those occurring at 9p21, 3p or 17p, as progression markers in HNSCC.

The first validation of the potential clinical utility of these markers was provided by Mao *et al.*²³. They performed microsatellite analysis at 9p21 and 3p14 on biopsies from patients with oral leukoplakia that had been enrolled in a retinoid/beta-carotene clinical chemoprevention trial - hence, outcome was known. Remarkably, both LOH events were related with early processes of tumorigenesis in HNSCC. Fifty-one percent of patients showed either or both alterations. Of these, 37% progressed to cancer. In contrast, only 1 patient (6%) progressed without either of these events.

Bringing LOH markers to community

With mounting interest in validation of these progression markers, the next challenge was to increase access to a suitable patient population for retrospective and prospective analyses. The British Columbia Oral Cancer Prevention Program (BC-OCPP) in British Columbia, Canada, used NIH/NIDCR funds to develop an infrastructure to facilitate this activity (Figure 1). For over a decade, the team has streamlined the referral pathway from community to specialized centres and engaged in research, including technology development that can be quickly applied and scaled-up for practical use. This infrastructure arose as a clinical research hub that, along with addressing social and behavioral determinants of disease, has addressed issues of quality in care and used linkages between community dental practices and a BC referral network to improve access to patients for study. A flow of patients was established between community dental practices through a centralized pathology service, the British Columbia Oral Biopsy Service (OBS) -- that received biopsies from dentists and ENT surgeons across the province -- to five dysplasia clinics, where risk assessment and follow-up occurred. The accrual of these patients was further facilitated by a series of social network/knowledge transfer-guided processes that strengthened the screening activity in British Columbia and built a sense of partnership with referring clinicians. Between 1999 and the present, over 600 patients with dysplasia (but no prior history of oral cancer) were accrued from these clinics into an ongoing Oral Cancer Prediction Longitudinal (OCPL) study. Using this structure, a series of retrospective and prospective studies were initiated to develop and validate potential risk markers for oral dysplasia, the first being the aforementioned LOH markers.

The first retrospective study, published in 2000, was used to confirm and refine the key markers to be used in the prospective (OCPL) study²⁴. A data linkage between recent

patients enrolled in the OBS and the Cancer Registry of British Columbia was used to identify a set of cases with potentially malignant lesions with known outcome. Archival tissue was collected from 116 individuals with PML (39 hyperplasia and 77 mild/moderate dysplasia) with no prior history of oral cancer. DNA from microdissected lesions were analyzed for LOH markers on 7 chromosome arms (3p14.2; 4q26, 4q31.1; 8p21.3, 8p23.3; 9p21; 11q13.3, 11q22.3; 13q12.3–13, 13q14.3; 17p11.2, and 17p13.1), the majority of which had been previously identified as promising candidates by the Johns Hopkins group. Among the best predictors of outcome from this analysis was LOH at 3p14 and/or 9p21, thus confirming the previously discussed work of Li Mao and co-workers²³. The data showed that lesions could be separated into profiles associated with low (LR) and high risk (HR) of progression, using only a few markers in these regions. Virtually all (28 out of 29) progressing cases showed LOH for one or both of these regions, with a greater than 20-fold increase in progression risk for lesions with 3p and/or 9p LOH compared with lesions with retention of both regions. Approximately 30% of high-risk (HR) lesions progressed to cancer in 5 years. Equally important, only 1 out of the 49 low-risk (LR) cases underwent progression.

In 2012, a data analysis of the first 296 patients with mild or moderate dysplasia in the ongoing prospective (OCPL) was published²⁵. At that time, patients had been followed for a median of 44.6 months (25th and 75th percentiles, 29.3 and 63.9), during which time 41 (13.9%) of them underwent progression, 17 to severe dysplasia, 2 to carcinoma in situ, and 22 to SCC (Figure 2A). Data analysis confirmed that histology alone did not significantly predict outcome (Figure 2B).

LOH status was determined using the same primers as the retrospective study. The study validated the model developed in 2000, with the HR profile (3p and /or 9p LOH) showing a 22.6-fold increase in risk compared to the LR profile (3p and 9p retention). A refinement of this model was developed within this cohort with the addition of another 2 markers (loci on 4q/17p) that further improved the risk prediction, with 5-year progression rates of 3.1%, 16.3%, and 64.1% for the LR, IR (intermediate risk), and HR lesions, respectively (Figure 2C). The HR group for this second model, identified by LOH on 3 arms (9p, 4q, and 17p), showed a 52.1-fold increase in progression risk compared with 9p retention alone (considered as a new LR profile). The predictive value of this refined model, which was called gMART (for genomic marker-based test) to better identify it for further study, was then reverse validated by using data from the previous retrospective cohort. Finally, a multi-covariate analysis that included key clinical and histological features demonstrated that LOH is the most significant predictor of progression, even in such combinations.

The clinical significance of gMART is that it can be used to improve the ability to target mild and moderate dysplasia with a high probability of progressing within short time spans (time to progression is significantly shorter for HR cases (see Figure 2C and D for 2, 3 and 5 year progression rates for LR, IR and HR profiles). The HR cases (LOH on 9p, 4q and 17p; 10.1% of cases) would be strong candidates for chemoprevention studies while LR cases (9p retention, 46.8% of informative cases) could be spared from needless monitoring and treatment, optimizing use of resources and removing individuals who would not benefit from chemopreventive studies. Intermediate-risk (IR) cases could either receive increased

surveillance or go to chemoprevention. As an extension of this research, we are exploring further ways in which to separate out cases by progression risk in the IR group (43.2% of cases) through either integration of other genetic markers to the gMART model or by integrating approaches other than genetics (see below).

ADDITIONAL APPROACHES

There is a growing recognition of the need for further tools and markers that build on the current gold standard -- oral dysplasia -- to help clinicians further separate such lesions by probable outcome. Table 1 provides examples of studies using an array of different approaches -- some use rapidly evolving new technologies, such as optics for tissue visualization or computer driven quantitative imaging for histology assessment. Other sources of putative markers come from new molecular and cellular technologies, such as those being created in parallel with next-generation sequencing.

Visualization with an early contrast agent

Toluidine blue (TB) is an interesting example of a putative marker that has a long history of use in the dental community -- and is now going through evaluation in the OCPL study as a potential marker of lesion behavior (Figure 3A). TB shows a fairly high sensitivity for the detection of oral cancers and high-risk dysplasia when applied to the oral mucosal surface using a swab ^{26, 27}. The ability to detect potentially malignant lesions is controversial, with a significant portion of dysplasia not staining with the dye. In 2005, Zhang *et al.* ²⁸ used the first 100 patients in the ongoing OCPL study to explore the possibility that the dye could preferentially stain lesions at high risk for malignant progression (Figure 3B). A 6-fold elevation in cancer risk was observed for TB-positive lesions, with 12 of 15 progressing lesions staining positive for the dye. Moreover, a multivariate analysis revealed an added value when TB was integrated with LOH, especially in predicting risk and outcome for PML with minimal or no dysplasia. Interestingly, these relationships hold true for both low- and high-grade dysplasia. It should be noted that although TB is an affordable and potentially useful tool for predicting progression of lesions with known oral dysplasia, there still exists challenges, particularly regarding specificity and accuracy, particularly in differentiating low-grade from hyperkeratotic lesions, which has prevented TB from being approved by the U.S. Food and Drug Administration for oral cancer screening ²⁹.

As contrast agents evolve as a potentially powerful technique for lesion visualization, it is likely that they will be integrated with molecular findings so that they are able to label specific molecular alterations associated with the neoplastic process. As such they may play a major role in identification and follow-up of oral mucosal sites with the highest risk of progression for biopsy and follow-up.

Fluorescence Visualization

A second form of visualization involves the use of optical devices that examine interactions of light with tissue. Interest in the use of optical changes (specifically, loss of fluorescence) in the epithelium in and around oral cancers has been centered on its use as a clinically applicable approach to map the "field of cancerization." ³⁰ Real-time visualization

approaches use hand-held devices to directly detect alterations associated with morphologic and biochemical changes to tissue during cancer development^{31,32}. In dysplasia clinics, fluorescence visualization has proven valuable in the detection of high-risk lesions, in the delineation of surgical margins and in follow-up after treatment³³. While this technology has significant promise, current devices are used only as an adjunct to conventional white light examination not as a “stand alone” tool, largely due to issues of specificity. In this context, the evolution of optical technology is ongoing with several modifications in development to improve both the diagnostic sensitivity and to reduce confounding associated with change in inflammatory conditions^{34–37}. These improvements, together with lowering costs of this technology, will further facilitate the use of these approaches in regular clinical practice.

High-resolution computer imaging systems

To address pitfalls from traditional histology diagnosis (such as low intra-observer and inter-observer agreement in grading epithelial dysplasia), and to objectively determine the weight to be associated with each of the visual characteristics^{21,38}), quantitative tissue pathology (QTP) uses computer-driven imaging systems to assist in judging the progression risk of PML. The association of DNA quantity and risk has been reproducibly demonstrated by several groups using independent cohorts^{39–42}, with evidence that combining such analysis with dysplasia grading gives a higher predictive value than either technique alone. In 2008, Guillaud *et al.*,⁴³ reported on the use of an algorithm (nuclear phenotype score, NPS) for identifying textural changes in oral lesions (such as heterochromatin versus euchromatin organization) and nuclear morphology changes (diameter, radius, and nuclear boundaries) that strongly associated with progression risk in a set of 69 PMLs. Individuals with a high NPS had a 10-fold increase in relative risk of progression. This algorithm has recently been validated prospectively on archival samples from oral dysplasia in the OCPL study (manuscript in preparation).

Genetic and epigenetics markers

Despite efforts made since the mid-1990s, there is a need for the development of more sophisticated markers to examine this subgroup of individuals to further stratify the risk (and to increase the specificity)²⁵. Only a few gene-specific markers have been reported to improve prediction of progression risk for patients with oral dysplasia. Examples generally use immunohistochemistry to compare gene expression in progressing to non-progressing lesions. Examples include podoplanin, Np63, pChk2, EZH2, SMAD4 and EGFR and others (see Table 1 for summary). It is anticipated that integration of data from the numerous high-throughput and high-resolution platforms able to identify different ‘omics dimensions (such as mutations, copy-number alterations, methylation, protein changes) will unwrap new alternatives to further optimize identification of progression markers. Epigenetic changes able to predict malignant progression in PML have been mostly described in the promoter region of *p16*^{44,45}. Also, a microRNA signature, including increase in hsa-miR-21, hsa-miR-181b and hsa-miR-345 expression, was identified as showing promise in ability to distinguish between progressive and non-progressive leukoplakia⁴⁶.

Combination of approaches

Table 1 also shows several studies that have used combinations of markers to assess risk. Lee *et al.* (2000) ¹⁴ reported an increase in predictive value when chromosomal polysomy, p53 expression, LOH and histology were combined. Saintigny *et al.* ⁴⁷ showed improvements in oral cancer risk prediction with combinations of podoplanin, Np63 and intraepithelial inflammatory cells. Studies with the OCPL cohort have shown that a combination of TB staining and LOH increases the capacity to delineate mucosal areas with high molecular risk, compared to each test alone ²⁸. Finally, the combination of LOH (3p/9p markers) and QTP increases the ability to detect lesions with both high molecular risk and the subtle histological change associated with outcome, thus enhancing overall predictive value against either approach alone ⁴³. Analysis of a validation of the latter approach has just been completed in a new group of patients in the OCPL study. That analysis has demonstrated that the combined algorithm is capable of identifying those lesions that will progress to cancer with ~80% sensitivity, while defining a low-risk group in which 0–2% of the lesions progress (manuscript in preparation).

THE FUTURE: CHALLENGES AND OPPORTUNITIES

As has been demonstrated in this article, it is possible to collectively develop a significant (and growing) number of promising markers to identify (with varying success) the risk of progression of PML. It is expected that this trend will continue, given the application of cutting edge technologies such as next-generation sequencing. This scenario is highly promising; however, lessons delivered from the first cycle (1996-now) of approaches used in this field suggest that the identification and development of new markers and their effective clinical implementation need to advance in parallel. In order to reach this objective, several key challenges remain.

Although these studies have expanded knowledge on progression markers, the data has been largely captured via retrospective analyses of cases identified in hospital archives, sometimes with chart review for case information and outcome. Thus detailed information on clinical attributes, lesion characteristics, and follow-up are often limiting. In some instances, devices have been identified (or optimized by further training on samples with known outcome) during the course of prospective studies and validation in a separate cohort of such patients is still ongoing. This effort needs to be continued.

Harmonized cohorts

One of the main aspects requiring attention is the availability and annotation of clinical samples used in studies. Since only a small proportion of cases progress to cancer, the ability of individual groups to collect critical samples is limited. The use of retrospective studies of cases with known outcome is a move in the right direction. As yet unexplored is the feasibility of cross validating the more promising findings from single studies via partnerships among individuals controlling samples from such retrospective cohorts. In addition, network development prior to study design will be a critical factor to allow for a harmonization of data collection in retrospective studies that will facilitate the fusion of data in meta-analyses. Such a step forward would help identify markers and tools that should be

evaluated in prospectively collected cohorts. The latter can then serve as the ultimate validation of selected predictive markers that show a strong, reproducible predictive value across multiple cohorts.

Development of research partnership with community

It is important to explore new ways in which to generate patient flow for accrual to prospective studies. This is not a trivial point, as such efforts require long-term partnerships and shared vision among large numbers of individuals and this has been a major problem for chemoprevention studies to date. The OCPL study used partnerships with the dental community and clinicians in the referral pathway (oral medicine specialists, pathologists and surgeons) to accrue the oral dysplasias for study. The creation of such partnerships in other venues needs to be explored. Knowledge translation and social marketing constructs can provide useful mechanisms for framing and nurturing these interactions.

Continued integration of new technology

As indicated in this review, the development and integration of technology at different levels can drive change by increasing knowledge of natural history of early disease and its consequence. This can lead to a better prediction of lesion behavior. Equally important, the sharing of such technology tends to build partnerships (e.g., between community and research centres, among researchers and clinicians, between industry and research). This can facilitate patient accrual and funding sources for prospective studies. It also opens a dialogue around clinical barriers between clinicians and researchers that can lead to further technology development.

A further obstacle is the difficulty in transferring validated tools and technology to end-users globally. For example, despite prospective evidence of the value of LOH for predicting risk of oral dysplasia the highly-specific technical expertise required for sample processing and analysis required for this assay made its implementation on a wider scale difficult. To this end, creation of robust versions of promising tools and their further evaluation is critical and the commercialization of such tools is important (reviewed in ²⁷).

CONCLUSION

The use of retrospective cohorts with known outcome for biomarker/tool development is a promising, yet challenging approach to a problem that has plagued chemoprevention studies in the oral cavity since its inception. It could speed up considerably the development and validation of promising markers that could better guide future chemoprevention studies. The evolution of this approach to an actual impact on patients will require an opening of a broader dialogue across research groups globally. This would create the potential for both cross validation of research findings in retrospective studies and for construction of prospective studies of sufficient size to validate the best markers. The fact that these studies are occurring now in multiple locations globally (e.g., North America, Europe, China, Japan, see references in Table 1) is significant. One of the biggest challenges to this evolution in approach is the creation of the necessary knowledge translation partnerships and networks to maximize the use of limited resources and to drive the process forward.

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Figure 1. British Columbia's system-wide approach to oral cancer

Efforts to improve referral pathways and pursue research are coupled with public education, strategies to foster linkages among professionals, institutions, and organizations, and mobilization of those networks to enhance care in under-served communities and improve professional practices for screening, referral and treatment. KT = knowledge translation; SES = socioeconomic status.

Figure 2. Predictive power of the new genome marker based test (gMART)

Risk prediction as performed in the prospectively validated cohort (N=296)²⁵. **A**) Overall progression of cohort. **B**) Probability estimation with histology alone: Hyperplasia (H), but with dysplasia history (red, N = 34), Mild dysplasia (red, N = 127), and Moderate dysplasia (green, N = 135). **C**) Probability of no progression evaluated as determined by LOH of markers on 9p, 4q and 17p. **D**) Progression rates for 2 (red), 3 (grey), and 5 (blue) years of follow-up using gMART model in retrospective (left) and prospective (right) cohorts. The gMART model was subsequently validated in the retrospective cohort originally described in Rosin et al., 2000²⁴.

Figure 3. Toluidine blue as a progression marker for potentially malignant lesions

A) Differences observed in lesions unstained (left) and stained (right) with toluidine blue (TB). TB staining offers different advantages in terms of visualization, selection of biopsy site and identification of surgical margins. B) TB-positive cases show a significantly lower rate of progression when compared with TB-negative cases (results obtained in a cohort of 100 cases). These data are undergoing validation in a separate cohort, alone and in combination with other markers).

Table 1

Different approaches and tools used to evaluate progression of potentially malignant lesions

Approaches	Description	References
Clinical lesion	Evaluation of lesion presence and clinical features (i.e. size, texture)	48
Histology	Presence and degree of dysplasia	
Tissue visualization	Contrast agents (Toluidine blue)	28
	Optics (Fluorescence visualization)	7
Computer imaging / Quantitative pathology	Changes in DNA amount (DNA ploidy/quantity, DNA index (flow cytometry))	39–42
	Quantitative measurement of nuclear features	43
Genetic changes	DNA dosage alterations	
	- <i>LOH at 3p &/or 9p</i>	22–24, 49
	- <i>LOH at 9p/17p/4q (gMART)</i>	25
	- <i>Copy numbers (determined by FISH): CCND1, EGFR and centromeres</i>	50
	Gene-specific and pathways alterations	
	- <i>Tetraspanins CD9 and CD151, EGFR, COX-2</i>	51
	- <i>Np63 expression</i>	47
	- <i>Podoplanin expression</i>	52, 53
	- <i>EZH2 expression</i>	54
	- <i>S100A7 expression</i>	55
	- <i>Stem cell regulation: ABC2, BMI-1 expression</i>	56
	- <i>SMAD4 expression</i>	57
	- <i>Phospholipase C-gamma 1 expression</i>	58
Epigenetic changes	- <i>Phosphorylated p120-catenin expression</i>	59
	- <i>DSPP or BSP expression (SIBLING family proteins)</i>	60
	Gene expression profile	61
Combinations	Genomic imbalance / Chromosomal instability	62, 63
	Gene-specific alterations	
	- <i>p16 promoter methylation</i>	44, 45
Combinations	microRNAs expression changes	
	- <i>miR21, miR181b, miR345</i>	46
	Chromosomal polysomy, p53, LOH & Histology	14
Combinations	Podoplanin, Np63 and EIC combined	47
	TB and LOH	28

Approaches	Description	References
	QP and LOH	43

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