

# Toluidine Blue Staining Identifies High-Risk Primary Oral Premalignant Lesions with Poor Outcome

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## Abstract

There is a pressing need for the development of visual aids that will facilitate the detection of oral premalignant lesions (OPLs) with a high-risk of progression. Preliminary data suggest that toluidine blue stain may be preferentially retained by OPLs with high-risk molecular clones. In this study, we monitored OPLs from 100 patients without any history of oral cancer for an average of 44 months in order to evaluate the association of toluidine blue status with clinicopathologic risk factors, molecular patterns (microsatellite analysis on seven chromosome arms: 3p, 9p, 4q, 8p, 11q, 13q, and 17p) and outcome. Toluidine blue-positive staining correlated with clinicopathologic risk factors and high-risk molecular risk patterns. Significantly, a >6-fold elevation in cancer risk was observed for toluidine blue-positive lesions, with positive retention of the dye present in 12 of the 15 lesions that later progressed to cancer ( $P = 0.0008$ ). This association of toluidine blue status with risk factors and outcome was evident even when the analysis was restricted to OPLs with low-grade or no dysplasia. Our results suggest the potential use of toluidine blue in identifying high-risk OPLs. (Cancer Res 2005; 65(17): 8017-21)

## Introduction

Despite refinement of surgical techniques and adjuvant therapies, the prognosis for patients with oral squamous cell carcinoma (SCC) remains poor with a 5-year survival rate (40-50%) that has not changed significantly for several decades (1). Early detection of oral premalignant lesions (OPLs) is central to the improvement of this prognosis. However, this detection relies heavily on the clinician's ability to differentiate such lesions from reactive and inflammatory conditions. Even when OPLs are identified, our ability to predict outcome is a challenge because the majority of OPLs will not progress. The presence of dysplasia, the current gold standard, is a good predictor of high-grade lesions but has only a limited capacity to predict outcome for lesions with minimal or no dysplasia, which constitute the majority of OPLs.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Toluidine blue staining is considered to be a sensitive adjunct tool for identifying early oral SCC and high-grade dysplasias (2-5). However, the detection of low-grade (mild/moderate) oral dysplasia has been less consistent, with a significant portion of such lesions not staining with toluidine blue (3, 5). Recent reports have associated toluidine blue retention in oral lesions with the presence of high-risk molecular clones, even in lesions with minimal or no dysplasia (6, 7), raising the possibility that toluidine blue could identify those low-grade lesions that are more likely to progress. In this study, we monitored 100 patients with primary OPLs to relate their toluidine blue status to outcome as well as to conventional histopathologic features and molecular risk patterns.

## Materials and Methods

**Patients.** The study group was chosen from 162 consented patients in longitudinal follow-up at the Oral Dysplasia Clinic, British Columbia Cancer Agency between 1996 and 2004. This is a referral center for oral dysplasia in the greater Vancouver area. Criteria for eligibility included: a history of histologically confirmed oral dysplasia but no history of head and neck cancer, the availability of a biopsy since enrollment with concurrent assessment of toluidine blue status for that biopsy (called the index lesion), and finally at least 6 months follow-up after the latter biopsy. Of these cases, 62 had <6 months of follow-up.

Of the 100 patients remaining, 47% were male, 69% had a smoking history, and 78% were Caucasian, with the rest being Asian and others. The average age was 59 (34-93 years). Index lesions were assessed for toluidine blue status using a topical application of 1% toluidine blue (OraTest) and destaining with acetic acid (1%) as previously described (4). Lesions were biopsied and histologic diagnosis confirmed by three head and neck pathologists (R. Priddy, K. Berean, and L. Zhang). Subsequently, toluidine blue status, lesion appearance and size were evaluated at 6-month intervals. The mean follow-up time for the 100 OPLs was 44 months, with more than half followed for  $\geq 3$  years (56%), and 27%  $\geq 5$  years. Of the 100 cases, four were lost to follow-up (two toluidine blue-positive and two toluidine blue-negative).

**Assessment of molecular risk pattern.** Areas of hyperplasia and dysplasia were microdissected from the index biopsies for microsatellite analysis. The underlying stroma was also collected as a source of matched control DNA. All samples were coded so that loss of heterozygosity (LOH) analysis was done without knowledge of diagnosis. The microsatellite markers mapped to the following regions: 3p14.2 (*D3S1234*, *D3S1228*, *D3S1300*); 4q26 (*FABP2*); 4q31.1 (*D4S243*); 8p21.3 (*D8S261*); 8p23.3 (*D8S262*); 8p23.3 (*D8S264*); 9p21 (*IFNA*, *D9S171*, *D9S1748*, *D9S1751*); 11q13.3 (*INT2*); 11q22.3 (*D11S1778*); 13q12.3-13 (*D13S170*); 13q14.3 (*D13S133*); 17p11.2 (*CHRN1*) and 17p13.1 (*tp53* and *D17S786*). These were markers used in previous studies to predict cancer risk of OPLs (8-13). The protocol for digestion and extraction of samples, LOH analysis, and scoring is described in Zhang et al. (14).

**Statistical analysis.** Differences and associations between groups (e.g., toluidine blue-positive versus toluidine blue-negative) were examined using either Fisher's exact test for categorical variables or *t* test for

continuous variables. We used event charts to evaluate the history of the patients (15). Time-to-progression curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. Relative risks and the corresponding 95% confidence intervals (95% CI) were determined using Cox regression analysis. All tests were two-sided.  $P < 0.05$  was considered to be statistically significant.

To test the hypothesis that toluidine blue-positive OPLs with low-grade dysplasia or no dysplasia have higher cancer risk than those histologically similar but toluidine blue-negative lesions, these lesions were also examined independently using the above statistical methods.

## Results and Discussion

We monitored 100 patients with primary OPLs to test for associations between toluidine blue status and outcome as well as to conventional clinicopathologic features and molecular risk patterns. Based on toluidine blue staining of the index lesion, the cases were classified as toluidine blue-positive ( $n = 36$ ) and -negative ( $n = 64$ ; Table 1). Patients with toluidine blue-positive and -negative OPLs were similar for gender (42% versus 50% male), ethnicity (83% versus 81% Caucasian), and smoking history (64% versus 72%; all comparisons showed  $P > 0.05$ ). The average age of patients with toluidine blue-positive lesions was 64 years, whereas the toluidine blue-negative lesions averaged 57 years ( $P = 0.005$ ).

**Toluidine blue preferentially stains lesions with clinical features associated with risk.** A number of retrospective studies suggest that three clinical features of primary OPLs are predictive of progression for OPLs: location on the floor of mouth, and ventrolateral tongue (termed high-risk sites); large size; and a nonhomogeneous clinical appearance (16–18). We examined 100 cases for association of toluidine blue stain with these features (Table 1).

A higher proportion of toluidine blue-positive lesions were located at high-risk sites (69% versus 53%); however, the difference was not significant ( $P = 0.14$ ). Toluidine blue-positive lesions tended to be larger than toluidine blue-negative lesions. This size difference was not significant at the beginning of the study (mean dimension  $19 \pm 15$  versus  $14 \pm 10$  mm,  $P = 0.16$ ); however, during follow-up, more toluidine blue-positive lesions grew in size compared with negative lesions, and the size difference became significant ( $27 \pm 19$  versus  $18 \pm 13$  mm,  $P = 0.0049$ ). Finally, toluidine blue staining was significantly associated with a nonhomogeneous clinical appearance: a higher number of nonhomogeneous OPLs were toluidine blue-positive both at the beginning of the study (59% versus 24% in toluidine blue-negative lesions,  $P = 0.0015$ ) and during follow-up (83% versus 41%,  $P < 0.0001$ ). These results suggest that toluidine blue preferentially stains high-risk OPLs as judged by the traditional clinical risk variables.

**Toluidine blue-positive oral premalignant lesions increase in frequency with histologic progression.** Toluidine blue uptake was associated significantly with degree of dysplasia, consistent with the literature. The stain was positive in 26% (5 of 19) of nondysplastic OPLs, 23% (15 of 64) of lesions with low-grade (mild/moderate) dysplasia, and 94% (16 of 17) with high-grade dysplasia ( $P < 0.0001$ ; Table 1). These results suggest that toluidine blue preferentially stains high-risk OPLs as judged by histologic variables. It should be noted that the 19 nondysplastic OPLs were judged clinically as nonreactive.

**Toluidine blue recognizes lesions with high-risk molecular patterns.** Toluidine blue-positive OPLs showed a consistently higher frequency of LOH for all seven chromosome arms, and four of these were significant: 3p, 9p, 11q, and 17p (all  $P < 0.05$ ;

**Table 1.** Clinical, pathologic, and molecular features of toluidine blue-positive and toluidine blue-negative OPLs

	All	Toluidine blue-negative	Toluidine blue-positive	<i>P</i>
Number of lesions	100	64	36	
Clinical features				
Located at high-risk sites (%)*	59 of 100 (59%)	34 of 64 (53%)	25 of 36 (69%)	0.14
Largest dimension (mean $\pm$ SD) at start of study	16 $\pm$ 12	14 $\pm$ 10	19 $\pm$ 15	0.16
Largest dimension (mean $\pm$ SD) during follow-up	21 $\pm$ 16	18 $\pm$ 13	27 $\pm$ 19	0.0049
Nonhomogeneous lesions at start of study (%)	34 of 100 (34%)	14 of 59 (24%)	20 of 34 (59%)	0.0015
Nonhomogeneous lesions during follow-up (%)	56 of 100 (56%)	26 of 63 (41%)	30 of 36 (83%)	<0.0001
Histologic features				
OPLs without dysplasia <sup>†</sup>	19	14 of 64 (22%)	5 of 36 (14%)	
OPLs with low-grade dysplasia	64	49 of 64 (77%)	15 of 36 (42%)	<0.0001
OPLs with high-grade dysplasia	17	1 of 64 (2%)	16 of 36 (44%)	
Molecular features				
OPLs with LOH	79	47 of 64 (73%)	32 of 36 (89%)	0.077
OPLs with LOH > 1 arm	41	17 of 64 (27%)	24 of 36 (67%)	0.0002
OPLs with LOH > 2 arms	27	7 of 64 (11%)	20 of 36 (56%)	<0.0001
Low risk pattern <sup>‡</sup> (retention of 3p and 9p)	43	35 of 64 (55%)	8 of 36 (22%)	0.0018
High-risk pattern <sup>‡</sup> (3p and/or 9p LOH plus other arm) <sup>§</sup>	33	9 of 64 (14%)	24 of 36 (67%)	<0.0001
Outcome				
OPLs progressing to cancer	15 of 100 (15%)	3 of 64 (5%)	12 of 36 (33%)	0.0002

\*Site at high-risk for cancer progression: floor of mouth and ventrolateral tongue.

<sup>†</sup>These patients had a history of oral dysplasia that was excised, but now had a hyperplastic lesion. For all of these cases, the lesion was at the former dysplasia site.

<sup>‡</sup>Based on Rosin et al. (10). Relative risk for low-risk was 1, and high risk 33.4.

<sup>§</sup>Includes loss at evaluated loci on 4q, 8p, 11q, 13q, or 17p.

Supplemental Table S1). In the context of previously established LOH patterns indicative of risk (8–13), toluidine blue staining was strongly associated with those LOH patterns with considerably increased cancer risk: multiple LOH ( $P < 0.0002$ ), and LOH at 3p and/or 9p plus losses at any other arm ( $P < 0.0001$ , Table 1); the later pattern has been associated with a 33-fold increase in risk of progression in a previous retrospective study of primary OPL (10, 11). These data support the ability of toluidine blue staining to delineate areas with high molecular risk.

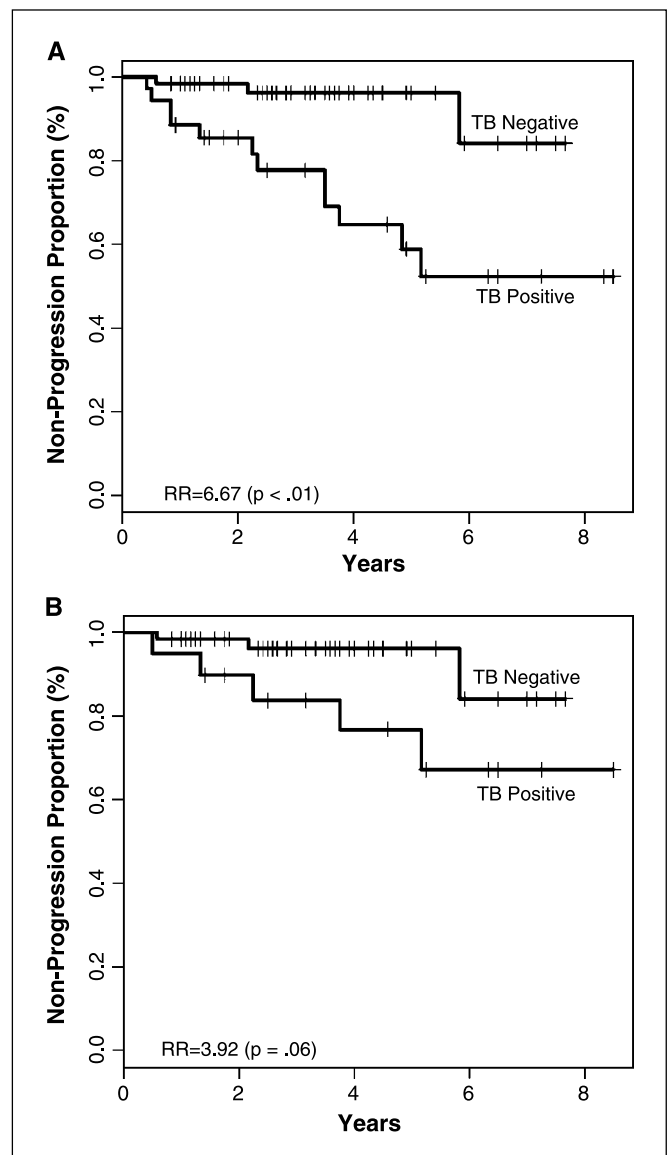
**Toluidine blue staining correlates with outcome.** The mean follow-up time for the 100 OPLs was  $44 \pm 26$  months. Within this follow-up period, 15 of the 100 OPLs progressed to oral SCC (from 4 of 19 hyperplasias, 4 of 64 low-grade dysplasias, and 7 of 17 high-grade dysplasias). The majority of the cancers (60%) developed at the same site as the index biopsy. The remaining 40% were adjacent to the index biopsy (within 2 cm). The average time for the OPLs to develop into SCC was  $30 \pm 22$  months ( $29 \pm 20$  for toluidine blue–positive lesions and  $34 \pm 32$  for toluidine blue–negative lesions). Only 3 (5%) of the 64 toluidine blue–negative OPLs progressed into SCC, whereas 12 (33%) of 36 toluidine blue–positive lesions developed into SCC ( $P = 0.0002$ ).

Time-to-development of SCC was significantly decreased for toluidine blue–positive when compared with the toluidine blue–negative lesions ( $P < 0.01$ , Fig. 1A). The hazard ratio based on the Cox regression for SCC development was >6-fold higher for toluidine blue–positive cases compared with the toluidine blue–negative cases (6.67; 95% CI: 1.87–23.70).

**Toluidine blue predicts risk and outcome for oral premalignant lesions with minimal or no dysplasia.** To test whether toluidine blue staining had value in identifying high-risk OPLs with little or no dysplasia, these lesions were examined independently. Of the 100 lesions, 83 belonged to the histologically low-risk lesions (19 hyperplasias and 64 low-grade dysplasias). Toluidine blue status and clinical and molecular features in this subgroup showed similar associations to those reported above (Table 2). Lesion size was not significantly different in the two groups at the beginning of the study (mean dimensions,  $18 \pm 16$  versus  $14 \pm 10$  mm;  $P = 0.74$ ). However, during follow-up, toluidine blue–positive lesions became significantly larger than the negative lesions ( $27 \pm 19$  versus  $18 \pm 13$  mm,  $P = 0.028$ ; Table 2). In contrast, a nonhomogeneous high-risk clinical appearance was more often apparent among toluidine blue–positive lesions, both at the beginning of the study (53% versus 22%,  $P = 0.02$ ) and during follow-up (75% versus 40%,  $P = 0.0097$ ).

Toluidine blue was retained at sites of high molecular risk lesions even among OPLs with little or no dysplasia. Again, an increase was observed for all chromosome arms in this subgroup, although differences in frequency for toluidine blue–positive and –negative lesions were not statistically significant (Supplementary Table S1). However, when the high-risk molecular patterns were compared, toluidine blue staining again was found to be associated with the high-risk molecular pattern: LOH at 3p and/or 9p plus any other arm (40% versus 14%,  $P = 0.023$ ; Supplementary Table S1).

Finally, toluidine blue staining and outcome for this subgroup was examined. Of the 83 OPLs with low-grade dysplasia or no dysplasia, 8 (10%) progressed into oral SCC (4 hyperplasias, 2 mild dysplasias, and 2 moderate dysplasias). Only 3 of the 63 (5%) toluidine blue–negative OPLs progressed into SCC compared with 5 of 20 (25%) toluidine blue–positive lesions ( $P = 0.0177$ ). Time-to-development of SCC was shorter for toluidine blue–positive lesions but the estimate was based on the small number of events and was



**Figure 1.** Probability of development of cancer from primary OPLs, according to toluidine blue–staining pattern. *A*, progression as a function of toluidine blue–staining capacity for all 100 OPLs (toluidine blue–positive, 36; toluidine blue–negative, 64). *B*, progression as a function for toluidine blue staining for OPLs without dysplasia or with low-grade dysplasia (severe dysplasia excluded; toluidine blue–positive, 20; toluidine blue–negative, 63).

only close to significance ( $P = 0.06$ , Fig. 1B). The relative risk for cancer progression was almost 4-fold higher for toluidine blue–positive OPLs (relative risk, 3.92; 95% CI, 0.92–16.80).

**Temporal analysis of toluidine blue status.** Figure 2 displays an event chart that summarizes the clinical time course of the OPLs in this study with respect to toluidine blue status and outcome. The date of the first toluidine blue assessment was set as time 0, and the time to treatment (either with excision or topical bleomycin), to subsequent cancers, or the last follow-up visit are shown. Also shown are dates at which an alteration in toluidine blue status occurred. When the toluidine blue status did not change during follow-up, the time event bar for that case remained unmarked.

For those lesions that were toluidine blue–negative at study entry, the majority (52 of 64, 81%) remained negative. Of interest, the three toluidine blue–negative cases that developed into cancer

**Table 2.** Risk features in OPLs with minimal or no dysplasia

	All	Toluidine blue–negative	Toluidine blue–positive	<i>P</i>
Number of lesions	83	63	20	
Clinical features				
Located at high-risk sites (%) <sup>*</sup>	44 of 83 (53%)	33 of 63 (52%)	11 of 20 (55%)	1
Largest dimension (mean ± SD) at start of study	15 ± 12	14 ± 10	18 ± 16	0.74
Largest dimension (mean ± SD) during follow-up	20 ± 15	18 ± 13	27 ± 19	0.028
Nonhomogeneous lesions at start of study (%)	23 of 83 (28%)	13 of 58 (22%)	10 of 19 (53%)	0.02
Nonhomogeneous lesions during follow-up (%)	40 of 83 (48%)	25 of 62 (40%)	12 of 20 (75%)	0.0097
Molecular features				
OPLs with LOH	62	46 of 63 (73%)	16 of 20 (80%)	0.77
OPLs with LOH > 1 arm	25	17 of 63 (27%)	8 of 20 (40%)	0.28
OPLs with LOH > 2 arms	13	7 of 63 (11%)	6 of 20 (30%)	0.072
Low risk pattern <sup>†</sup> (retention of 3p and 9p)	43	35 of 63 (56%)	8 of 20 (40%)	0.31
High-risk pattern <sup>‡</sup> (3p and/or 9p LOH plus other arm) <sup>‡</sup>	17	9 of 63 (14%)	8 of 20 (40%)	0.023
Outcome				
OPLs progressing to cancer	8 of 83 (10%)	3 of 63 (5%)	5 of 20 (25%)	0.0177

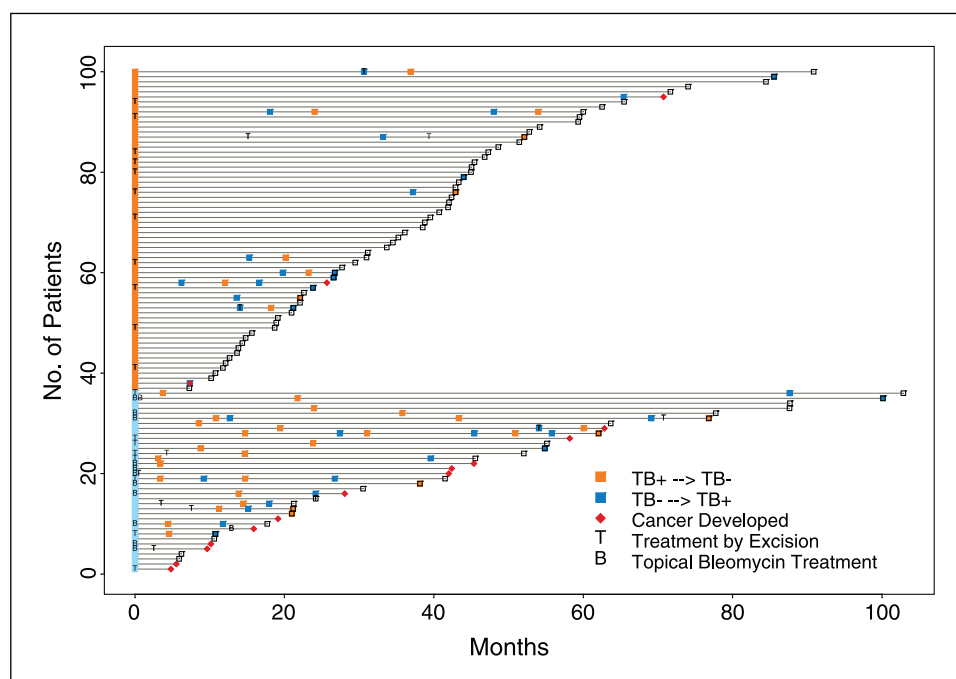
\*Site at high-risk for cancer progression: floor of mouth, ventrolateral tongue.

<sup>†</sup>Based on Rosin et al. (10). Relative risk for low-risk was 1, and high risk 33.4.

<sup>‡</sup>Includes loss at evaluated loci on 4q, 8p, 11q, 13q, or 17p.

were predicted by a change in toluidine blue status. In two cases, this transition in status was detected 6 and 15 months prior to diagnosis. For the third case, the cancer developed quickly within 8 months of the initial evaluation, with a change to toluidine blue–positive leading to the diagnostic biopsy of SCC. Of the remaining 10 toluidine blue–negative lesions which showed a transition to positive staining, two returned to negative after treatment and four were found at the last visit. The remaining four cases showed a fluctuation of the staining between toluidine blue–positive and toluidine blue–negative over time, possibly due to a biopsy effect.

In contrast, only 1 of 36 (3%) toluidine blue–positive lesions became negative without apparent intervention. Five toluidine blue–positive lesions (14%) became toluidine blue–negative either after treatment (two lesions), or after incisional biopsy (three lesions). The majority of toluidine blue–positive lesions (30 of 36, 83%) remained positive continuously (15 cases) or intermittently (15 cases) throughout follow-up. In the latter case, the transition to a toluidine blue–negative status followed an incisional biopsy (2 of 15, 13%) or, more frequently, treatment with an intent to cure (10 of 15, 67%; 8 surgery and 2 bleomycin). These lesions later showed a



**Figure 2.** A time event chart showing (a) treatment, (b) changes in toluidine blue status during follow-up, and (c) outcome. Each case was labeled for its toluidine blue status at the beginning of the study (orange for toluidine blue–negative and blue for toluidine blue–positive). Treatment: excision (T), topical bleomycin (B). Change in toluidine blue status during follow-up: ■, change of toluidine blue staining from toluidine blue–negative to toluidine blue–positive; ■, change of toluidine blue staining from toluidine blue–positive to toluidine blue–negative. When the toluidine blue status had not changed, e.g., started as toluidine blue–negative and remained negative during the whole follow-up time, the time event bar for that case would remain unmarked. Outcome: □, last follow-up time for lesions that had not progressed. This square was colored blue or orange when there was a change in toluidine blue status on that date. ♦, cancer development.

reversion to toluidine blue-positive status, and in two of these cases, the later development of cancer. These data illustrate the difficulty of managing such lesions, with frequent re-emergence of the toluidine blue-positive phenotype with time after treatment. The results strongly suggest the necessity of continuous monitoring of dysplastic lesions, even posttreatment, and especially those with a history of toluidine blue retention.

Cancer development is a complex process. Currently, we have little understanding of factors affecting the speed of cancer transformation for high-risk lesions judged by either gold standard histology or molecular markers. For example, our previous study has shown that some OPLs with the high-risk LOH pattern (33-fold increase in cancer risk) took 8 years to become cancer, whereas others took only 6 months (10). Similarly, whereas toluidine blue staining showed a predictive value for cancer transformation, the time to transformation differed greatly as shown by the wide range of time interval between emergence of toluidine blue-positive lesions and tumor progression (Fig. 2). Nonetheless, like molecular markers, toluidine blue staining could provide clinicians with an additional tool for judging cancer risk of OPLs and guide the management of these lesions (e.g., monitoring or intervention with surgery and/or chemoprevention) before we understand the factors that could trigger the transformation of these high-risk lesions.

**Clinical translation potential.** Our data support the potential value of using toluidine blue as an adjunct tool for clinical

diagnosis of high-risk primary OPLs in a high-risk clinic (a referral center for dysplasias). Not only did toluidine blue detect virtually all of the high-grade dysplasia (16 of 17 cases) in this study, but it also preferentially stained OPLs with minimal or no dysplasia with high-risk clinical and molecular attributes. Moreover, the staining status was strongly associated with outcome. Admittedly, the study benefited from the involvement of Oral Medicine and Pathology specialists who were experienced in both the use of the dye and in clinical assessment of OPLs, reducing confounding false-positive staining of reactive or inflammatory lesions such as denture trauma. Given these promising results from this pilot study, the efficacy of this stain in predicting outcome for primary OPLs needs to be further evaluated within a clinical trial scenario, next in a community setting and with a larger cohort. However, the significance of the study is that it points to a need to re-access toluidine blue stain not just with its association with histology, but also with molecular risk predictors and with outcome.

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