In this issue of Clinical Cancer Research, Zeng et al. (1) describe the analysis of putative intermediate biomarker gene expression in oral cavity mucosa biopsies from 17 patients with advanced malignancies who were enrolled in a Phase I clinical trial of liarozole. This agent was administered p.o. at three doses (75, 150, and 300 mg twice daily), and biopsies were collected from the oral cavity mucosa at baseline and after 4 weeks of treatment. Total RNA was analyzed for the expression of the intermediate biomarkers EGF-R, TGF, transforming growth factor receptor; TGF, transforming growth factor; RAR, retinoic acid receptor; RT-PCR, reverse transcription-PCR; ATRA, all-trans-retinoic acid; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; RA, retinoic acid.

Liarozole, a novel benzimidazole derivative, is a potent inhibitor of cytochrome P450-dependent 4-hydroxylation of endogenous ATRA, thereby increasing ATRA levels in both plasma and certain tissues (2, 3). Because prolonged use of RAs leads to side effects, Zeng et al. (1) have chosen to use liarozole to determine whether it can elevate endogenous ATRA, thereby increasing ATRA levels in both plasma and certain tissues (2, 3). In human epidermis, topical liarozole enhanced responses to retinol or ATRA by inhibiting 4-hydroxylase (6). Similarly, oral administration of liarozole at 300 mg 1 h before an oral dose of ATRA (45 mg/m²) partially reversed the decline in ATRA level (due to oxidation) observed in patients treated with ATRA as a single agent (7). Thus, an improved strategy for using liarozole in clinical trials may be to combine it with ATRA.

Biomarkers related to cancer can be defined as phenotypic or genotypic characteristics that are altered during carcinogenesis and/or during response to chemopreventive or therapeutic intervention. Such biomarkers can be used for detection, differential diagnosis, assessment of disease, and assessment of efficacy and toxicity of preventive or therapeutic agents. Because intervention trials that rely on cancer development or recurrence or on patient survival as end points are very prolonged and may require large populations, biomarkers are needed to serve as surrogate or intermediate end points. Such biomarkers should substitute for clinical end points such as decreased cancer incidence, delay of recurrence, or increased survival. Ideally, the surrogate end point is on the pathway of cancer development and is modulated by intervention. Biomarkers that change during the carcinogenesis process can indicate how far a lesion has advanced. Other biomarkers can indicate whether a chemopreventive or therapeutic agent has reached the target tissue and induced one or more cellular or molecular effects predicted on the basis of the agent’s mechanism of action. Biomarkers that are restored to normal expression level by a chemopreventive or therapeutic agent can indicate potential response to the intervention. To establish that a biomarker is a valid surrogate end point requires large clinical trials using cancer development as an end point in chemopreventive trials and recurrence or metastasis as an end point in therapeutic adjuvant trials. However, at present, no biomarker has been fully validated. Therefore, in the meantime, we have to use unvalidated biomarkers as surrogates for clinical efficacy. Understanding the biology of the intermediate biomarkers in the oral mucosa of the liarozole-treated patients is that liarozole failed to increase endogenous ATRA levels to those required to affect the expression of the biomarkers. Previous studies have shown that liarozole failed to inhibit the proliferation of breast and prostate carcinoma cell lines or to enhance the differentiation of mouse embryonal carcinoma cells in vitro (2, 3). In patients with NSCLC, liarozole (300 mg twice daily p.o.) caused side effects usually seen with ATRA but was well tolerated. However, no objective tumor response was seen in any of the 14 patients with stage IIIIB and IV NSCLC (4). In vitro studies have shown that in breast and embryonal carcinoma cells, liarozole enhanced the effects of exogenous ATRA on growth inhibition and differentiation (2, 3). Likewise, a combination of ATRA and liarozole showed a 10× greater induction of the RAR-β2-lacZ reporter (5). In human epidermis, topical liarozole enhanced responses to low levels of retinol or ATRA by inhibiting 4-hydroxylase (6). Similarly, oral administration of liarozole at 300 mg 1 h before an oral dose of ATRA (45 mg/m²) partially reversed the decline in ATRA level (due to oxidation) observed in patients treated with ATRA as a single agent (7). Thus, an improved strategy for using liarozole in clinical trials may be to combine it with ATRA.

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cancer may help in the identification of such markers. Indeed, Zeng et al. (1) have based their choice of biomarkers on their previous demonstration that both EGF-R and its ligand TGF-α are elevated in HNSCCs and in histologically normal mucosal samples from patients with HNSCC compared with control normal mucosa from patients without cancer. Furthermore, Grandis et al. (8–10) have shown that both EGF-R and TGF-α could be down-regulated by ATRA in HNSCC cell lines. The intermediate biomarker RAR-β was chosen because its mRNA levels have been reported to decrease during oral carcinogenesis even in premalignant lesions and to be up-regulated by 13-cis-RA in vivo (11). Likewise, cellular RA-binding protein II has been implicated in ATRA metabolism and response to ATRA (12, 13).

The choice of these biomarkers appears to be plausible because they have been modulated by ATRA in vitro and in vivo. They were not increased by liarozole in oral mucosa biopsies, possibly because the ATRA concentration required to modulate these biomarkers is higher than that achieved by liarozole treatment. Other studies have shown that the levels of one of the biomarkers used here, RAR-β, could be increased in vivo in patients treated with pharmacological doses of 13-cis-RA (11, 14).

Analysis of expression of intermediate biomarkers is best accomplished with tissues that are accessible directly for biopsy or brushing (e.g., premalignant and malignant tissues in skin, oral cavity, cervix, bladder, and colon) or with secretions (sali-

va, sputum, bronchial lavage, and bladder washing) or body fluids (blood and urine). However, many cancers arise in tissues that are inaccessible. It has been proposed that some accessible tissues may serve as surrogates for inaccessible tissues. Re-

cently, Kopelovich et al. (15) have suggested that cancer risk in inaccessible target organs can be assessed by analysis of accessible surrogate tissues. They proposed that a requirement from a surrogate tissue is that it be in either anatomical continuum or functional (mechanistic) continuum (if noncontiguous) with the inaccessible organ. Their assumption is that genetic changes that can be detected in the surrogate site may predict risk in the inaccessible site. It is not clear whether the concept can also be applied to modulation of biomarkers by chemopreventive or therapeutic agents because those responses depend on uptake of the agents, metabolism of the agent, and regulation of expression of different genes that may be distinct in different tissues. For example, Ayoub et al. (14) compared RAR-β mRNA expression in brushings from the oral cavity (palate) and bronchi from 40 smokers and found complete agreement in only 68% of the subjects. Thus, even in an epithelium that is continuous and exposed to the same carcinogenic insult, there is substantial difference in the expression of a specific biomarker. Sun et al. (16) have shown that NSCLC cell lines are generally resistant to the growth-inhibitory effects of ATRA, whereas many HNSCC cell lines are susceptible (17). Thus, response to ATRA in the oral cavity may not predict response in the lung. Zeng et al. (1) used oral cavity mucosa biopsies as surrogate tissue to assess response to liarozole among patients with advanced malignan-

cies at sites that were not specified in the report. Therefore, it is not possible to comment on the suitability of the oral biopsy to serve as a surrogate for those types of malignancy. The use of oral mucosa biopsies as a surrogate for tumors that develop in organs such as the breast or prostate may have limitations because the mechanisms of carcinogenesis in these organs are distinct from the oral mucosa in terms of the etiology and the differentiation and proliferation signaling pathways that are abrogated during carcinogenesis.

Zeng et al. (1) have demonstrated the analysis of RNA isolated from whole biopsies by quantitative RT-PCR assay, using a primer dripping method to measure the expression level of biomarkers relative to a control housekeeping gene. They recommend that methods suitable for small quantities of RNA should be considered and devised for use in the analysis of small biopsies. Castillo et al. (18) have previously described a fast and sensitive method based on combining RT-PCR with a colori-

metric ELISA. They have demonstrated the use of this method for the analysis of RAR-β mRNA using total RNA extracted from small laryngeal biopsies and related it quantitatively to a β2 microglobulin internal control (18). In their method, PCR products are labeled with digoxigenin during amplification, and the amplified DNA is hybridized with a biotinylated probe. The hybrid is then captured on a streptavidin-coated microtiter plate, and detection is accomplished using antidigoxigenin antibodies conjugated with peroxidase and a peroxidase substrate (18). The methods described by both Zeng et al. (1) and Castillo et al. (18) can be improved by a combination of laser capture microdissection under direct microscopic visualization (19) and real-time PCR (20). By extracting total intact RNA from the whole biopsy without first separating the minor subpopulation of epithelial cells from the stromal cells, Zeng et al. (1) may have missed changes in biomarker(s) that are expressed differentially at baseline or after liarozole treatment in epithelial and mesenchymal cells. For example, ATRA has been reported to up-regulate EGF-R in fibroblasts (21) but to decrease EGF-R in epithelial cells (10). Laser capture microdissection under direct micro-

scopic visualization permits rapid one-step isolation of selected cell populations from a section of heterogeneous tissue. This technique has been used successfully in conjunction with PCR amplification of RNA from dissected cells (19). The real-time quantitative PCR method measures PCR product accumulation by following a dual-labeled fluorogenic probe (i.e., TaqMan Probe) progressively during the exponential phase of the reaction so that quantitative PCR is accomplished. This method provides very accurate and reproducible quantitation of transcripts (mRNA), even if the starting amount of RNA is in the nanogram range, as in the present study. Real-time quantitative PCR is extremely accurate and is less labor intensive than current quantitative PCR methods because it can be automated and used for high throughput analysis of multiple samples (20, 22).

In conclusion, the development of quantitative methods to improve the use of intermediate end point biomarkers to predict response to therapy is clearly an important endeavor. Zeng et al. (1) have described one method to analyze material extracted from small biopsies for expression of intermediate end point biomarkers, and other improvements are suggested above. These methods can be applied for the very important assessment of response to chemopreventive and therapeutic agents in the short term. However, long-term studies with clinical end points are needed to validate specific biomarkers and the use of accessible surrogate tissues to avoid equivocal results.
References


Are We Ready to Use Surrogate End Points and Surrogate Tissues to Evaluate Response to Chemopreventive and Therapeutic Intervention?

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