# Effect of Duration of Fixation on Quantitative Reverse Transcription Polymerase Chain Reaction Analyses

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**Increasingly, there is the need to analyze gene expression in tumor tissues and correlate these findings with clinical outcome. Because there are few tissue banks containing enough frozen material suitable for large-scale genetic analyses, methods to isolate and quantify messenger RNA (mRNA) from formalin-fixed, paraffin-embedded tissue sections are needed. Recovery of RNA from routinely processed biopsies and quantification by the polymerase chain reaction (PCR) has been reported; however, the effects of formalin fixation have not been well studied. We used a proteinase K-salt precipitation RNA isolation protocol followed by TaqMan quantitative PCR to compare the effect of formalin fixation for 24, 48, and 72 hours and for 1 week in normal (2), oral epithelial dysplasia (3), and oral squamous cell carcinoma (4) specimens yielding 9 fresh and 36 formalin-fixed samples. We also compared mRNA and protein expression levels using immunohistochemistry for epidermal growth factor receptor (EGFR), matrix metalloproteinase (MMP)-1, p21, and vascular endothelial growth factor (VEGF) in 15 randomly selected and routinely processed oral carcinomas. We were able to extract RNA suitable for quantitative reverse transcription (RT) from all fresh (9/9) and formalin-fixed (36/36) specimens fixed for differing lengths of time and from all (15/15) randomly selected oral squamous cell carcinoma. We found that prolonged formalin fixation (>48 h) had a detrimental effect on quantitative RT polymerase chain reaction results that was most marked for MMP-1 and VEGF but less evident for p21 and EGFR. Comparisons of quantitative RT polymerase chain reaction and immunohistochemistry showed that for all markers, except p21, there was good correlation between mRNA and protein levels. p21 mRNA was overexpressed in only one case, but protein levels were elevated in all but one tumor, consistent with the established translational regulation of p21. These results show that RNA can be reliably isolated from formalin-fixed, paraffin-embedded tissue sections and can produce reliable quantitative RT-PCR data. However, results for some markers are adversely affected by prolonged formalin fixation times.**

**KEY WORDS: Cancer, Fixation, Formalin, PCR, Quantification, Real time, Reverse transcription.**

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A number of methods are available to analyze messenger RNA (mRNA) expression in tissues including Northern hybridization, subtractive hybridization, RNase protection assay, mRNA differential display, and cDNA microarrays. All have the potential to characterize differentially expressed genes providing new insights into the biology of many diseases. However, these methods are significantly limited by the requirement for fresh, unfixed tissues to permit isolation of abundant, high-quality mRNA.

Although many institutions are building frozen tissue banks, few are at the stage to permit large-scale genetic analyses or to have sufficiently long-term follow-up to yield meaningful clinical data. By contrast, there is a vast supply of archived formalin-fixed, paraffin-embedded tissue blocks, often with long patient follow-up and outcome data (1). The ability to predictably recover sufficient RNA for cDNA template generation and subsequent quantitative polymerase chain reaction (PCR) thus offers tremendous potential in the study of a myriad of


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diseases. Potentially, quantitative PCR offers a number of advantages over traditional methods because it permits the use of relatively small amounts of genetic material that may be fragmented or degraded, such as that obtained from routinely processed tissue sections (2).

Recent studies have demonstrated that RNA can be extracted from formalin-fixed, paraffin-embedded tissue sections, converted to cDNA, and then subjected to quantitative PCR (3, 4). Comparisons of fresh and fixed tissues using quantitative PCR methods have shown that mRNA expression levels are similar, supporting the validity of this method for analysis of gene expression in fixed tissues (5). Some studies have examined the effect of differing formalin fixation times ranging from 12 to 24 hours and have found that formalin fixation does not adversely affect the quality of RNA that is destined for PCR analysis (6–9). These short fixation times may be applicable to small biopsies collected in hospital settings but likely do not reflect the more typical prolonged fixation times that are used for larger specimens or those collected in outpatient settings. Therefore, the objective of this study was to assess the effect of prolonged formalin fixation on the suitability of mRNA for quantification using the real-time TaqMan PCR assay.

## MATERIALS AND METHODS

### Tissue Collection and Processing

For testing of the effect of differing fixation times on tissues for quantitative reverse transcription polymerase chain reaction (RT-PCR), biopsies of normal oral mucosa ($n = 2$), oral epithelial dysplasia ($n = 3$), and oral squamous cell carcinoma ($n = 4$) were obtained from the Oral Cancer Research Center tissue bank at the University of California San Francisco. All tissues had been obtained at the time of surgical excision, immediately snap frozen in liquid nitrogen, and stored at −70° C. Each sample was then thawed briefly and divided by scalpel into five similarly sized pieces. To minimize contamination, a new scalpel was used for each specimen. To ensure proper and rapid fixation, all tissue pieces were sectioned to ensure that all spatial dimensions were ≤5 mm (5 × 5 × 5 mm). One piece (0-h sample) was placed in RLT buffer from the Qiagen RNA extraction kit (Qiagen Inc., Valencia, CA). The other four pieces were immediately placed in 10 mL of 10% buffered formalin and fixed for 24, 48, and 72 hours and for 1 week. After formalin fixation, samples were dehydrated, incubated in xylene, and then embedded in paraffin using a Leica Paraffin Embedder (Leica Microsystems Inc., Deerfield, IL). There were a total of 9 fresh samples and 36 formalin-fixed specimens. All diagnoses were confirmed by examination of 5-μm hematoxylin and eosin–stained sections to ensure that all tissues were similarly representative.

To compare mRNA and protein expression, we used routinely processed paraffin sections of 15 oral squamous cell carcinoma obtained from the tissue bank. All specimens were similarly sized and had been immediately fixed in 10% neutral buffered formalin for 24 hours before being processed to paraffin as described above.

### RNA Isolation from Fresh Tissues

RNA was isolated from fresh tissues using the Qiagen RNeasy Total RNA System (Qiagen Inc.) according to the manufacturer's protocol. RNA was resuspended in 100 μL of RNase-free water. RNAs were quantified by spectrophotometer at OD of 260 nm, and A260/A280 ratios ≥ 1.8 were considered high purity (5).

### RNA Isolation from Fixed Tissues

Total RNA was isolated from 4 × 10–μm paraffin sections using modifications to the Paraffin Block RNA Isolation kit (Ambion Corp., Austin, TX). Briefly, sections were microtome cut from paraffin-tissue samples onto clean glass microscope slides. The microtome was cleaned after each use, and the tissue sections floated in a water bath containing DEPC-treated water before being mounted on glass slides. Sections were deparaffinized three times in xylene for 5 minutes each, followed by one wash with 100% ethanol for 5 minutes. The sections were then scraped off the glass slides using a clean razor blade and placed in 105 μL of RNA lysis buffer containing 500 μg/mL of proteinase K. Sections were then incubated for 18 hours, at 56° C, until the tissues were solubilized. RNA was obtained by extraction with an equal volume of 70% phenol (pH 4.3):30% chloroform at room temperature. Samples were centrifuged for 5 minutes at 15,000 rpm and the aqueous phase transferred to new, RNase-free Eppendorf tubes. The RNA was precipitated by the addition of an equal volume of isopropanol and 1 μg of linear acrylamide for 2 hours at −20° C. The samples were then centrifuged for 15 minutes at 14,000 rpm, and the pellets washed in 70% ethanol, air dried on the bench top, and then resuspended in 50 μL of RNase-free water. Ten microliters of extracted RNA was then treated with 1 μL DNase I (2U/μL) in 2 μL of 10× DNase I Reaction Buffer and 7 μL of Nuclease-free water and incubated for 15 minutes at 37° C. RNA was then extracted with acid phenol–chloroform and precipitated by adding 1 μL of linear acrylamide (5 mg/mL); 10 μL of 3 M sodium acetate, pH 4.5; and one volume of isopropanol, incubated in −20° C for 30 minutes and

resuspended in 50 μL of RNase-free water as the final volume.

### Reverse Transcription

RT was performed using Gibco BRL Reverse Transcription kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Reactions were carried out in 20-μL volumes consisting of 1× buffer, 200 U of Moloney-murine leukemia virus reverse transcriptase, 40 U/μL RNase inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), 5 μmol/L random hexamers (Life Technologies) and 5 μL total RNA. Reactions were incubated in a PCR thermocycler at 25° C for 10 minutes, 37° C for 50 minutes, and 70° C for 15 minutes and then cooled to 4° C. After RT, samples were diluted by adding 60 μL of purified water (Sigma, St. Louis, MO). Negative controls were "no mRNA," in which 5 μL of RNase-free water was substituted for mRNA template in the RT reaction.

### Real-Time Quantitative RT-PCR

The relative abundance of epidermal growth factor receptor (EGFR), p21, matrix metalloproteinase (MMP)-1, and vascular endothelial growth factor (VEGF) mRNA was assessed using the 5' fluorogenic nuclease assay to perform real-time quantitative PCR. EGFR, MMP-1, and VEGF were selected because they have been shown to be overexpressed at the mRNA and protein level in a large proportion of oral squamous cell carcinoma (10–13). p21 protein is also elevated in large proportion of oral dysplasias and squamous cell carcinomas but shows no mRNA overexpression (14, 15).

The basis for the TaqMan system is continuous measurement of PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe. On the 5' terminus is a reporter dye (FAM = 6-carboxyfluorescein), and on the 3' terminus is a quenching dye (TAMRA = 6-carboxytetramethylrhodamine). The oligonucleotide sequence is homologous to a target sequence, and PCR primers encompassing the probe sequence are added. When the probe is intact, energy transfer between the two fluorophors occurs, and the quencher eliminates emission from the reporter. During the extension phase of PCR the probe is cleaved by 5' nuclease activity of *Taq* polymerase, releasing the reporter from the oligonucleotide quencher and resulting in an increase of reporter emission intensity. Intron-spanning primer and probe sequences (Table 1) either were obtained from published sequences (4, 5) or were designed to meet specific criteria by using Primer Express design software (PE Biosystems, Foster City, CA; 16). Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA).

The ABI 7700 Prism (PE Biosystems) uses fiber optics connected with each well in a 96-well PCR tube arrangement so as to laser excite and measure the fluorescence spectra intensity from each tube with continuous monitoring during PCR amplification. Each tube is reexamined every 8.5 seconds. Computer software examines the fluorescence intensity of reporter and quencher over the course of the amplification so as to calculate continuously the increase in normalized reporter emission intensity. This result was plotted *versus* time, represented by cycle number, to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot was examined at a point during the log phase of product accumulation accomplished by assigning a fluorescence threshold above background and determining the point at which each amplification plot crosses the threshold (defined as the threshold cycle number, or Ct). Differences in Ct were used to quantify the relative



**TABLE 1. Sequences of PCR Primers and Fluorogenic Probes Used in Quantitative PCR**

| Gene (Reference No.) | Primer/Probe | Sequence | Amplicon Size (bp) |
|---|---|---|---|
| β-Gus (5) | β-Gus-F | CTCATTTGGAATTTTGCCGATT | 81 |
| | β-Gus-R | CCGAGTGAAGATCCCCTTTTTA | |
| | β-Gus-probe | FAM-TGAACAGTCACCGACGAGAGTGCTGG | |
| EGFR (5) | EGFR-F | CGCAAGTGTAAGAAGTGCGAA | 93 |
| | EGFR-R | CGTAGCATTTATGGAGAGTGAGTCT | |
| | EGFR-probe | FAM-CCTTGCCGCAAAGTGTGTAACGGAAT | |
| MMP-1 | MMP-1-F | GAGGGTCAAGCAGACATCATGA | 52 |
| | MMP-1-R | CAAGATTTCCTCCAGGTCCATC | |
| | MMP-1-probe | FAM-TGTCAGGGGAGATCATCGGGACAA | |
| p21 (4) | p21-F | CTGGAGACTCTCAGGGTCGAA | 66 |
| | p21-R | GGCGTTTGGAGTGGTAGAAATCT | |
| | p21-probe | FAM-ACGGCGGCAGACCAGCATGA | |
| VEGF (5) | VEGF-F | CTCTACCTCCACCATGCCAAG | 90 |
| | VEGF-R | AGACATCCATGAACTTCACCACTTC | |
| | VEGF-probe | FAM-TGGCAGAAGGAGGAGGGCAGAATCA | |

All probes were labeled with 3′TAMRA (6-carboxytetramethylrhodamine) quencher dye. PCR, polymerase chain reaction; FAM = 6-carboxyfluorescein; EGFR, epidermal growth factor receptor; MMP-1, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

amount of PCR target contained within each tube. Assuming that each reaction functions at 100% PCR efficiency, a difference of one Ct represents a 2-fold difference in the amount of starting template.

Quantitative RT-PCR was performed in triplicate 50-$\mu$L reaction volumes consisting of 1× PCR buffer (PE Biosystems), 5.5 mmol/L $MgCl_2$, 0.5 $\mu$M of forward and reverse primers, 0.2 $\mu$M of probe, 0.025 U/$\mu$L AmpliTaq Gold (PE Biosystems), and 5 $\mu$L of the appropriate RT reaction. Two-step PCR cycling was carried out as follows: 95° C for 12 minutes (1 cycle), 95° C for 15 seconds, 60° C for 1 minute (40 cycles). At the end of the PCR, baseline and threshold values were established using the ABI 7700 Prism software, and the Ct values were exported to Microsoft Excel (Microsoft Corp., Redmond, WA) for analysis.

### Calculation of Relative Expression

Relative expression of mRNA was calculated using the comparative Ct method previously described (5, 17). This method of analysis was selected because the slopes of the standard curve for each marker (Fig. 1) were not significantly different across a range of input RNA, thus differences in relative abundance for low- or high-quantity RNA species would not distort the analysis. Moreover, absolute Ct values for each sample were found to all lie within the range of Ct values used for standard curve generation.

Analysis was carried out using the sequence detection software supplied with the ABI 7700 (PE Biosystems). This software calculates the Ct for each reaction and uses it to quantify the amount of starting template in the reaction. All data were controlled for quantity of input RNA by performing measurements on an endogenous reference gene $\beta$-*N*-acetyl-glucuronidase ($\beta$−Gus; 5, 18). The Ct values for each set of three reactions were averaged for all subsequent calculations. A difference in Ct values ($\Delta$Ct) was calculated for each marker by taking the mean Ct of triplicate tubes and subtracting the mean Ct of triplicate tubes for $\beta$−Gus. To calculate the relative RNA of a test sample compared with normal RNA, a $\Delta$Ct was determined as follows:

$$\Delta Ct = Ct\ (EGFR) - Ct\ (\beta - Gus)$$

From this value, a relative RNA expression can be calculated using the following formula:

$$\text{Relative RNA} = 2^{-\Delta Ct}$$

Using this calculation, we defined no change in relative RNA expression as when this value was equal to 1; overexpression, when it was >1; and under expression, when it was <1. For simplicity, PCR efficiencies were assumed to be 100%. PCR efficiencies were measured as previously described(16) and were found to be >95% (Fig.1); therefore, this assumption introduced minimal error into the calculation.

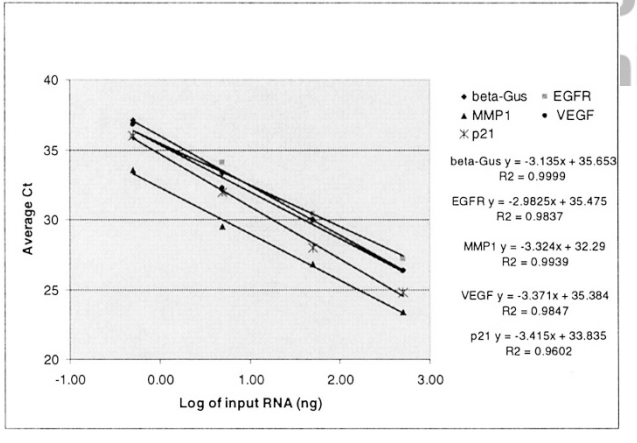


**FIGURE 1.** The logarithm of the input RNA amount from the same sample plotted *versus* the threshold cycle (Ct) monitored during TaqMan quantitative RT-PCR. Amplification efficiency of $\beta$-*N*-acetyl-glucosaminidase, epidermal growth factor receptor, matrix metalloproteinase, vascular endothelial growth factor, and p21 genes are shown as regression lines. All points represent the mean of duplicate PCR amplifications. Error bars are too small to be shown.

### Immunohistochemistry

Immunohistochemistry was performed as previously described (19) to determine the expression levels of EGFR, MMP-1, p21, and VEGF proteins. Briefly, 5-$\mu$m sections were cut and mounted on adherent glass slides (Fisher Scientific, Houston, TX). Sections were dewaxed in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity was blocked by immersion in 0.5% methanolic peroxide for 15 minutes, followed by two washes in 1× phosphate buffered saline (PBS) for 5 minutes each. Immunoreactivity of the target antigen was enhanced using pressurized heat antigen retrieval using 0.6 M sodium citrate buffer and heating to 100° C for 15 minutes (20). The sections were then removed, quenched in deionized water, and rinsed in PBS. The sections were then incubated for 1 hour at room temperature with primary antibodies to EGFR, MMP-1, p21, or VEGF diluted to 1:100 in PBS. Monoclonal anti-EGFR (Clone 528) was obtained from Oncogene Research Products (Cambridge, MA) and recognizes the external domain of the epitope. Monoclonal anti-MMP-1 (Clone 41–1E5) was obtained from Oncogene Research Products and recognizes both latent (57/52 kDa) and active (46/42 kDa) forms of MMP-1. Monoclonal anti-p21 (Clone EA-10) was obtained from Biogenex (San Ramon, CA). Polyclonal anti-VEGF (Clone A-20) recognizes 165, 109, and 121 amino acid isoforms and was obtained from Santa Cruz Bio-

technology (Santa Cruz, CA). Incubation was followed by two washes in PBS and then application of a biotinylated secondary antibody, washed twice in PBS followed by the application of preformed avidin–biotin complex (DAKO Corp., Carpinteria, CA) for 30 minutes (21). The bound complexes were visualized by the application of a 0.05% solution of 3-amino-9-ethylcarbozole (Sigma Corp.) containing 0.3% hydrogen peroxide as a substrate. After incubation, the sections were washed and then lightly counterstained in hematoxylin and coverslipped.

### Quantification of Immunohistochemistry

Immunohistochemical staining was scored by examining sections at 40× objective to assess the proportion of cells showing membrane (EGFR), cytoplasmic (MMP-1, VEGF), or nuclear (p21) expression and with confirmation of positive staining done at 100× objective. The percentage of positive cells was scored using the method of Xia *et al.* (22) as follows: 3, >50% of cells staining +; 2, 25 to 49% of cells staining +; 1, 5 to 24% of cells staining +; 0: negative or <5% of cells staining +.

## RESULTS

### RNA Extraction

We were able to isolate mRNA from 9/9 (100%) fresh and 36/36 (100%) tissue biopsies of normal, epithelial dysplasia and squamous cell carcinoma fixed for differing times. In addition, we were also successfully able to extract RNA and amplify cDNA from 15/15 (100%) routinely processed oral squamous cell carcinoma obtained from the tissue bank.

### Quantitative mRNA Analysis in Fresh Tissue

By quantitative RT-PCR, we found that both cases of normal epithelium did not show overexpression of EGFR, MMP-1, or VEGF but that there was a mean 2-fold relative increase in p21 mRNA (Table 2). For the dysplasia and squamous cell carcinoma groups, mean mRNA expression levels for MMP-1, EGFR, p21, and VEGF were elevated. By immunohistochemistry, overexpressed (≥2+) EGFR protein was seen in none of the normal epithelial controls, 2 of 3 dysplasias, and 3 of 4 squamous cell carcinomas. Two carcinomas showed overexpressed (≥2+) p21 protein, and two squamous cell carcinoma showed MMP-1 protein overexpression (≥1+). VEGF was overexpressed (≥2+) by all oral squamous cell carcinomas.

### Effect of Formalin Fixation Time on Quantitative RT-PCR

Results of fixation time are summarized in Table 3, where values have been normalized to the fresh tissue (time = 0 h). Values represent the relative change in mRNA expression in comparison to the expression levels in fresh, unfixed tissues. Therefore, a value of 1 indicates no change in expression between the fixed specimens and fresh tissue at that time point. These results are summarized graphically in Figure 2. In general, prolonged formalin fixation (>48 h) had a detrimental effect on the amplification efficiency of PCR. These effects were variable and depended on the specific probe and the duration of fixation. For all markers studied, the results at 24 hours were similar to the case in fresh tissues. However, by 48 hours of fixation, both MMP-1 and VEGF expression appeared to be markedly increased compared with that seen in the fresh tissues. p21 showed markedly altered gene expression, compared with results from fresh tissues, at 72 hours. By contrast, EGFR showed relatively robust results at all time points up to 1 week. Signal was not detected for any marker in negative controls (Ct = 40).

### Quantitative mRNA Analysis in Archival Tumor Tissues

To further examine the suitability of the quantitative RT-PCR assay, we compared mRNA levels to protein levels in 15 archived, formalin-fixed, paraffin-embedded oral squamous cell carcinoma



**TABLE 2. Mean Relative mRNA Expression Levels of MMP-1, EGFR, p21, and VEGF in Fresh Tissue Biopsies of Normal, Dysplastic and Neoplastic Oral Epithelium**

| Factor | Normal Epithelium (*N* = 2) | | Dysplasia Epithelium (*N* = 3) | | | Squamous Cell Carcinoma Epithelium (*N* = 4) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sample No. | | Sample No. | | | Sample No. | | | |
| | 1 | 2 | 1 | 2 | 3 | 1 | 2 | 3 | 4 |
| MMP-1 | 0.13 | 0.02 | 0.07 | 8.96 | 1.84 | 2.07 | 0.66 | 24.50 | 5.10 |
| EGFR | 0.20 | 0.54 | 0.93 | 4.58 | 3.93 | 0.39 | 1.61 | 31.30 | 3.61 |
| p21 | 4.45 | 0.08 | 0.31 | 16.37 | 4.71 | 16.02 | 0.60 | 0.66 | 0.31 |
| VEGF | 1.00 | 0.02 | 0.44 | 88.44 | 0.01 | 10.29 | 0.22 | 151.17 | 3.54 |

All data points represent mean of triplicate runs. All points were measured relative to β-Gus. MMP-1, matrix metalloproteinase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

**TABLE 3. Summary of Changes in mRNA Expression Compared with Fresh Tissue after Differing Intervals of Fixation in 10% Buffered Formalin**

| Fixation Time[a] | Normal ($N$ = 2) | Dysplasia ($N$ = 3) | Carcinoma ($N$ = 4) | Mean |
|---|---|---|---|---|
| | | MMP-1 | | |
| 0 h | 1.00 | 1.00 | 1.00 | 1.00 |
| 24 h | 0.52 | 2.27 | 1.60 | 1.47 |
| 48 h | 0.51 | 9.29 | 4.14 | 4.65 |
| 72 h | 4.02 | 4.54 | 13.56 | 7.37 |
| 1 week | 8.92 | 3.07 | 1.80 | 4.60 |
| | | EGFR | | |
| 0 h | 1.00 | 1.00 | 1.00 | 1.00 |
| 24 h | 1.50 | 0.78 | 0.65 | 0.98 |
| 48 h | 1.43 | 0.52 | 0.76 | 0.91 |
| 72 h | 1.82 | 0.49 | 1.01 | 1.11 |
| 1 week | 1.38 | 0.62 | 0.98 | 0.99 |
| | | p21 | | |
| 0 h | 1.00 | 1.00 | 1.00 | 1.00 |
| 24 h | 0.56 | 1.22 | 1.04 | 0.94 |
| 48 h | 1.13 | 0.69 | 0.91 | 0.91 |
| 72 h | 6.01 | 0.73 | 0.87 | 2.54 |
| 1 week | 0.47 | 0.75 | 0.80 | 0.68 |
| | | VEGF | | |
| 0 h | 1.00 | 1.00 | 1.00 | 1.00 |
| 24 h | 0.86 | 2.16 | 4.98 | 2.67 |
| 48 h | 3.31 | 2.23 | 13.89 | 6.48 |
| 72 h | 10.21 | 0.60 | 36.44 | 15.75 |
| 1 week | 0.19 | 1.31 | 10.74 | 4.08 |

Results are the mean of triplicate runs. All points were measured relative to β-Gus and normalized to fresh tissue RNA. No change = 1. MMP-1, matrix metalloproteinase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

[a] 0 h = fresh, unfixed.

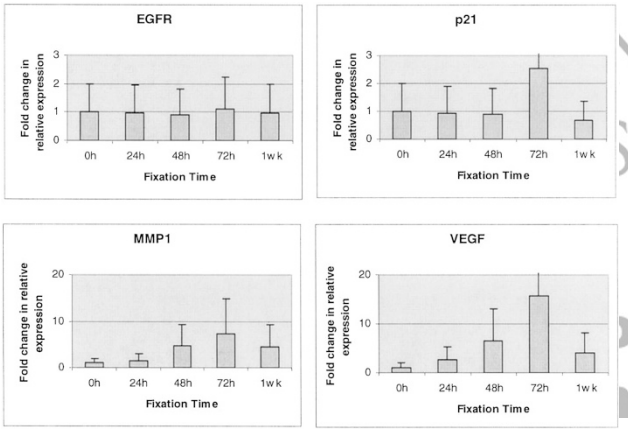


**FIGURE 2.** Effect of differing fixation times on quantification of epidermal growth factor receptor, p21, matrix metalloproteinase, and vascular endothelial growth factor mRNA species in oral tissues ($n$ = 9). ***Solid bars*** indicate the mean change in relative expression for each marker in the same tissues fixed for differing intervals after normalization to the expression in unfixed, frozen tissue (0 h); no change = 1. ***Error bars*** represent standard deviations. All points were measured relative to β-*N*-acetyl-glucosaminidase.

using the markers EGFR, MMP-1, p21, and VEGF. Results are shown in Table 4 and summarized in Table 5. We were able to isolate mRNA, transcribe to cDNA, and amplify by PCR in all (15/15; 100%) cases. RNA signal was not detected for any marker in negative controls.

We found EGFR mRNA overexpression (relative expression >1) in 14/15 (93%) oral squamous cell carcinoma, and one case showed reduced EGFR expression (relative expression <1). Overexpression of mRNA ranged from 1.3-fold to 9-fold. EGFR protein overexpression (≥2+) was identified in 10/15 (67%) tumors. In normal epithelium, EGFR protein was expressed only in basal keratinocytes, accounting for approximately 5% of the total keratinocyte population.

For MMP-1, there was mRNA overexpression in 14/15 (93%) oral squamous cell carcinoma and protein overexpression in the same 14/15 (93%) cases. Because MMP-1 protein was not seen in normal epithelial controls (data not shown), we interpreted MMP-1 protein levels ≥1+ (≥5% positive cells) as abnormally expressed (23). Similarly for VEGF, 14/15 (93%) cases showed increased mRNA, and 11/15 (73%) cases showed elevated protein levels. VEGF protein was weakly expressed in <5% of normal keratinocytes, and therefore we interpreted overexpression when there was ≥2+ staining. p21 mRNA was underexpressed in all cases, but by immunohistochemistry all but one tumor showed overexpressed protein.

All tumors showing overexpressed EGFR, MMP-1, or VEGF protein showed increased mRNA by real time-PCR. By contrast, no case showed p21 mRNA overexpression, and all but one case had elevated protein levels by immunohistochemistry.

**TABLE 4. Comparison of mRNA Expression Levels Determined by Quantitative RT-PCR Assay and Protein Expression by Immunohistochemical Analysis in Formalin-Fixed, Paraffin-Embedded Tissue Sections of Oral Squamous Cell Carcinoma**

| Specimen No. | Marker[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | EGFR | | MMP-1 | | p21 | | VEGF | |
| | mRNA | Protein | mRNA | Protein | mRNA | Protein | mRNA | Protein |
| 1 | 3.24 | 3 | 12.52 | 2 | 0.37 | 3 | 3.85 | 3 |
| 2 | 9.00 | 3 | 4.37 | 0 | 0.06 | 3 | 8.82 | 3 |
| 3 | 1.29 | 2 | 15.49 | 1 | 0.41 | 3 | 4.76 | 3 |
| 4 | 1.92 | 1 | 5.40 | 1 | 1.31 | 2 | 1.69 | 1 |
| 5 | 4.56 | 3 | 0.43 | 0 | 0.58 | 3 | 7.36 | 3 |
| 6 | 1.09 | 3 | 9.87 | 3 | 0.12 | 3 | 1.40 | 1 |
| 7 | 2.63 | 3 | 9.29 | 3 | 0.38 | 1 | 4.59 | 3 |
| 8 | 1.80 | 1 | 58.89 | 3 | 6.84 | 3 | 4.92 | 3 |
| 9 | 3.16 | 3 | 87.22 | 3 | 0.60 | 3 | 17.75 | 3 |
| 10 | 2.39 | 1 | 39.85 | 3 | 0.72 | 3 | 6.29 | 3 |
| 11 | 2.38 | 2 | 10.36 | 3 | 0.90 | 3 | 6.50 | 3 |
| 12 | 0.81 | 0 | 7.97 | 3 | 0.31 | 3 | 1.23 | 1 |
| 13 | 4.18 | 3 | 11.60 | 1 | 0.38 | 3 | 10.08 | 3 |
| 14 | 4.03 | 3 | 9.25 | 1 | 0.12 | 2 | 13.83 | 3 |
| 15 | 2.34 | 2 | 4.73 | 3 | 0.64 | 3 | 0.93 | 1 |

All samples fixed for 24 hours; results are the mean of triplicate runs. EGFR, epidermal growth factor receptor; MMP-1, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

[a] Percentage of positive cells for protein was scored as follows: 3, >50% of cells +; 2, 25 to 49% +; 1, 5 to 24% +; 0, negative or <5% of cells +.

**TABLE 5. Summary of mRNA Expression Levels Determined by Quantitative RT-PCR Assay and Protein Expression by Immunohistochemical Analysis in Formalin-Fixed, Paraffin-Embedded Tissue Sections of Oral Squamous Cell Carcinoma**

| Marker | mRNA Overexpression,[a] *n* (%) | Protein Overexpression,[b] *n* (%) |
|---|---|---|
| EGFR | 13 (87) | 10 (67) |
| MMP-1 | 14 (93) | 14 (93) |
| p21 | 2 (13) | 14 (93) |
| VEGF | 14 (93) | 11 (73) |

EGFR, epidermal growth factor receptor; MMP-1, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

[a] mRNA overexpression defined as cases showing relative expression >1.

[b] Based on expression in normal epithelium, overexpression of EGFR, p21 and VEGF proteins was defined as cases having a score of 2+ or greater (>25% positive cells). For MMP1 protein, there was no expression in normal epithelium so overexpression in tumors was defined as cases having a score of 1+ or greater (>5% positive cells).



## DISCUSSION

The purpose of this study was to examine the effect of prolonged formalin fixation on quantitative RT-PCR analysis. We found that mRNA can be reliably extracted from formalin-fixed, paraffin-embedded tissue sections but that prolonged formalin fixation (>48 h) has a detrimental effect on quantitative RT-PCR that is marker specific.

It is well established that DNA can be extracted from formalin-fixed, paraffin-embedded tissue sections and subjected to molecular analysis (24, 25). In the study of cancer, DNA analysis has proven useful for the study of molecular changes in tumors and for the identification of potential pathogens that may cause specific neoplasms (26–28). The molecular analysis of DNA extracted from paraffin sections is also a frequent component in the routine assessment of many types of lymphomas (29). However, there are a number of important biological limitations when studying DNA from tumors, and hence the analysis of transcriptional events, providing a dynamic assessment of the cells, is more desirable.

In the past, it was thought that RNA extracted from paraffin tissue sections was too degraded to permit analysis (30). Many early attempts to extract RNA from formalin-fixed, paraffin-embedded tissues yielded low quantity and poor-quality material. Technical problems to overcome include RNA fragmentation, formalin-induced protein cross-linking, the addition of monomethyl groups to nucleotides, dimerization of adenine which interferes with subsequent PCR, ubiquitous RNase enzymes, and paraffin interference with RNA extraction by guanidinium hydrochloride (1, 31).

More recent studies have shown that RNA can be extracted successfully from formalin-fixed paraffin-embedded tissue section and used for cDNA template generation and subsequent PCR (4, 5, 32, 33). The most effective isolation methods involve the use of a concentrated proteinase K digestion step to solubilize tissue proteins and reverse monomethyl nucleotide modification to RNA (24, 30). Although these methods can yield RNA suitable for PCR amplification and analysis, the effect of prolonged formalin fixation has not been well studied. Previous studies that have examined this issue in a controlled manner have used fixation times of ≤24 hours (3, 5, 24, 34, 35). Although this duration may be suitable for small tissues obtained in some hospital settings, it is likely shorter than those typically used for larger specimens or those obtained in outpatient settings or in mail-in biopsy services.

We used a modified proteinase K digestion protocol with a phenol extraction step for the isolation of RNA from formalin-fixed, paraffin-embedded tissue sections (4). We show that isolation of RNA and conversion to cDNA was possible for all formalin-fixed specimens, irrespective of fixation time. This finding is in agreement with those of others who have also shown reliable isolation of RNA from paraffin sections using proteinase K digestion (5, 6, 9, 30, 32, 36). Significantly, by coupling our extraction method with TaqMan RT-PCR, we were able to generate reproducible quantitative data. It has been shown previously that PCR success was limited by attempts to amplify large target fragments and that best success was obtained when target amplicons were small (5). Therefore, we used primers designed to generate amplicons of <100 bp in size. Our results, therefore, show that the extraction of RNA, conversion to cDNA, and quantitative PCR analysis should be possible in the majority of cases using the protocol we describe here.

It is unclear why there were differences in the performance of different markers using the same study material. Both MMP-1 and VEGF showed marked alterations in their expression levels by 48 hours, in contrast to EGFR and p21, which showed more robust amplification results. One possibility may be due to differences in amplicon sizes, although this seems unlikely because all targets were <100 base pairs in size. Moreover, the EGFR amplicon size was the largest marker at 93 base pairs and yet performed well on all material irrespective of fixation duration. It is conceivable that results may be related to differences in mRNA sequences and the generation of secondary structures that are more susceptible to the effects of prolonged formalin-fixation. Furthermore, absolute Ct values were higher at 72 hours than at 1 week, suggesting that the amount of recoverable RNA was less at the earlier time point. We believe that this result is unlikely to be caused by differences in primer amplification efficiencies because the slopes of the standard curves for all markers relative to the reference gene were similar over a range of input RNA. Moreover, this is unlikely to be caused by processing because all specimens had been handled in an identical manner, apart from duration of formalin fixation. Therefore, our results suggest that although RNA yield from formalin-fixed, paraffin-embedded tissue sections is high, for accurate quantitative RT-PCR it is necessary to select primers judiciously and limit fixation times to 24 hours.

We found that in all cases of oral squamous cell carcinoma in which there was protein overexpression of MMP-1, EGFR, and VEGF, there was a corresponding overexpression of mRNA, as determined by the quantitative RT-PCR assay. These results were expected because these mRNA and protein species are recognized to be overexpressed in a significant proportion of oral squamous cell carcinoma (10). Some oral squamous cell carcinomas showed high expression levels of different mRNA species (*i.e.*, MMP-1 *versus* EGFR) but correspondingly low protein expression. This finding is not surprising and may be attributed to differences in translation of specific mRNA species in these tumors (37). By contrast, we found that p21 mRNA was frequently low in the oral squamous cell carcinoma but that p21 protein was often overexpressed. This finding is consistent with that of a previously published study comparing p21 mRNA and protein levels in oral squamous cell carcinoma (14) and supports the concept that p21 protein levels are primarily regulated by translational mechanisms through ubiquitin-mediated degradation (38). Thus, our results confirm the suitability of quantitative RT-PCR for determining relative mRNA expression in formalin-fixed, paraffin-embedded tissue sections.

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