ity in granulocytes is ~2.4-fold greater than that in mononuclear cells (Table 1). The clinical significance of these results will also likely be in the realm of heterozygosity testing. In addition, some individuals with α-iduronidase deficiency have undergone bone marrow or cord blood transplantation therapy (22, 27), and here, too, biochemical monitoring of enzyme activities in mixed leukocyte preparations could at times provide misleading results, suggesting the possibility of loss of engraftment when, instead, the distribution of types of leukocytes in the peripheral blood has changed.

In summary, this study provides data regarding six lysosomal enzymes that are frequently measured and shows that all six enzymes have significantly different activities per gram of cell protein in mononuclear cells compared with granulocytes. These data have clinical implications in the areas of biochemical diagnosis of patients having partial deficiencies of lysosomal enzyme activities, in heterozygosity testing, and in the monitoring of patients who have undergone bone marrow or cord blood transplantation.

References


Gene amplifications and deletions play an important role in the pathogenesis of solid tumors, including prostate cancer. Real-time PCR is a powerful tool for quantitative DNA analysis, particularly when starting quantities of tumor tissue are minimal (1–5). In the present study, we describe a modification of the 2-ΔΔCT method, which recently was shown to be suitable for relative gene expression analyses (6). We used our technique to analyze prostate cancer cell lines and tissue samples to determine the relationship between homeodomain-containing transcription factor 3.1 (NKX3.1) and MYC gene copy number alterations in this tumor type.

The specimens analyzed included (a) blood samples from healthy donors; (b) the prostate cancer cell lines DU145 (ATCC no. HTB-81) and PC3 (ATCC no. CRL-1435) and their derived sublines DU145M1, PC3-N, and PC3-125-1L (7); (c) the colorectal cell line COLO320DM (ATCC no. CCL-220), which harbors a high-level MYC amplification; and (d) primary prostate adenocarcinoma samples obtained after radical prostatectomy from previously untreated patients. The specimens were histologi-
cally verified, and samples were taken as described previously (8). We extracted DNA from the snap-frozen prostate carcinoma samples, blood samples, and cell lines, using the Blood and Cell Culture DNA Mini Kit (Qiagen).

We performed quantitative real-time PCR using the LightCycler™ system (Roche Diagnostics) with the FastStart DNA Master SYBR Green I LightCycler Kit (Roche Diagnostics). PCRs were run in duplicate with 20-μL reaction volumes containing 1× SYBR Green I PCR Buffer Mix, 5 mM MgCl2, 0.5 mM each primer, 1× FastStart Taq DNA Polymerase (Roche Diagnostics), and 50 ng of genomic DNA. The cycling conditions are given in Table 1. The hot start PCR method was applied to prevent incomplete DNA denaturation as discussed by Wilhelm et al. (9). Melting curve analysis was performed for each reaction to exclude nonspecific PCR side products. We analyzed the run data, using the Second-Derivative Maximum Method (LightCycler quantification software 3.5; Roche Diagnostics). The crossing point (CP) values, defined as the points at which the fluorescence increased appreciably above background fluorescence, were averaged for each set of two reactions. For each individual run, three dilutions of COLO320DM DNA were used to generate a gene-specific internal calibration curve. Before analysis of the prostate cancer samples, PCR amplification efficiencies (E) were determined according to the equation: \( E = 10^{(-1/slope)} \) (10). The calibration curves were created by use of five to eight dilutions containing 0.39–100 ng of COLO320DM DNA as input with three PCR repeats each (Fig. 1, A–C). The efficiencies were as follows: 1.97 for MYC, 1.99 for NKX3.1, and 1.99 for GAPDH, each with a high linearity [Pearson correlation coefficient (r) = −1.00].

The primers used are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is located at 12p13, served as reference gene. The region 12p13 has been shown to not be involved in prostate carcinogenesis. The existence of specific primer binding sites in pseudogenes of GAPDH was excluded by use of the BLASTN database.

As a modification of the 2^{−ΔΔCT} method, we calculated the relative gene copy number from the real-time PCR efficiencies, which were determined for each individual run, and the CP deviations of the target and reference genes in a test sample vs a control: \( E = \frac{CP_{target} - CPref_{control}}{CP_{target} - CPref_{pred}} \). We determined \( E \) for each individual run by constructing an internal gene-specific calibration curve with COLO320DM DNA. Because the gene copy number is 2 in normal diploid DNA, the relative copy number multiplied by 2 yields the copy number in the test sample for the target gene. Concerning the accuracy of our modified real-time PCR method, measurements of the MYC gene copy number in the prostate cancer cell lines revealed a high concordance with the previously reported fluorescence in situ hybridization results (7). We also found the method to be sufficiently sensitive to distinguish one copy from two copies. For this purpose, we determined the difference in copy numbers of the androgen receptor (AR) gene at chromosome Xq12 in healthy human males vs females (Fig. 1, D and E). Our analyses showed mean (SD) relative copy numbers of 1.18 (0.18) for male DNA and 2.27 (0.34) for female DNA. We observed no difference between males and females for the autosomal gene topoisomerase II isozyme β (TOP2B), located at 3p.

We determined the intra- and interassay variances of LightCycler runs by use of the COLO320DM cell line and DNA extracted from blood samples from healthy individuals. We investigated the interassay variance for the MYC gene in five repeats within one LightCycler run. We calculated interassay variance from three experimental runs performed on 3 successive days with three different master premixes. The mean variance was acceptable: 0.48 cycles in intertest experiments and 0.12 cycles in intratest experiments.

To demonstrate the utility of our modified quantitative real-time PCR method, we analyzed the NKX3.1 and MYC gene copy numbers in 35 primary prostate adenocarcinomas. The detailed findings are presented in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue3/. NKX3.1 and MYC were selected because they are supposed to play an important role in prostate carcinogenesis [for a review, see Ref.(11)]. The NKX3.1

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**Table 1. Sequences and annealing temperatures of the gene-specific primers.**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers</th>
<th>Sequences, 5′→3′</th>
<th>Length, bases</th>
<th>( T_m ), °C</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAPDH FWD</td>
<td>ACGTGTCAGTGTGGTAGACTG</td>
<td>21</td>
<td>59</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>GAPDH REV</td>
<td>AGTGCGTGCTTCGTTGAAAGT</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKX3.1</td>
<td>NKX3.1 FWD</td>
<td>CTCCCTCTGCTGCCTGCTGCT</td>
<td>21</td>
<td>68</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>NKX3.1 REV</td>
<td>CGGGAGGCGAAAGTAAGAAAGC</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYC oncogene</td>
<td>MYC FWD</td>
<td>GAGAAAAACAAAGAATGAGG</td>
<td>20</td>
<td>59</td>
<td>315</td>
</tr>
<tr>
<td></td>
<td>MYC REV</td>
<td>GTTCGCCCTCTGACATTTC</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP2B</td>
<td>TOP2B FWD</td>
<td>CAATTTTTCTGTCGCTGCTGCTG</td>
<td>20</td>
<td>60</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>TOP2B REV</td>
<td>GCTGAAATCTGCTGAAAGC</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>AR FWD</td>
<td>CGGAAGCTGAGAACTGG</td>
<td>20</td>
<td>58</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>AR REV</td>
<td>ATGGCTCTCAGGACATTCG</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Cycling conditions: initial activation, 95 °C for 10 min; template activation for 36 cycles with denaturation at 95 °C for 15 s, primer annealing for 5 s, and extension at 72 °C for 20 s.

*b* \( T_m \), melting temperature.
gene maps to 8p21. Authors of studies based on comparative genomic hybridization (CGH) and microsatellite/loss of heterozygosity analyses agree that 8p losses are one of the most common genetic changes in prostate cancer, with reported frequencies of 30–86% in different studies (12). Amplification and overexpression of the MYC protooncogene at 8q24 have been observed in 8% of primary prostate cancers, increasing to ~30% in advanced/metastatic tumor stages (13, 14).

To determine whether results obtained by real-time PCR analysis of the prostate cancer samples were significantly different from the mean results obtained for samples from healthy individuals, we determined a tolerance interval (TI) for the relative gene copy numbers, using the mean (SD) \( \Delta CP \) values for target and reference genes in 11 healthy individuals according to the equation: TI = 2 \( \pm \) (SD\( \Delta CP \) \times 2). The TI ranged from 1.44 to 2.56 for NKX3.1 and from 1.63 to 2.37 for MYC. To distinguish chromosome 8 aneusomy from gene-specific changes, we determined the TI for the copy number ratio of NKX3.1 to MYC; the resulting values ranged from 0.77 to 1.23. We compared the PCR results for the tumor tissue samples with the CGH findings on aliquots of the same DNA samples. CGH was performed as described elsewhere (7).

CGH detected loss of genetic material at 8p in 6 of 35 (17.1%) cases analyzed. This relatively low incidence of detected 8p losses contrasts to the 18 cases (51.4%) for which real-time PCR revealed a significant decrease in NKX3.1 gene copy number, from 1.39 to 0.20 copies/cell. Conversely, in three of the six cases with CGH-detected loss at 8p, real-time PCR detected no changes in the NKX3.1 gene copy number. CGH detected a gain in genetic material at 8q in 9 cases (25.7%), whereas PCR detected an increase in MYC in 13 cases (37.1%; from 2.45

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**Fig. 1.** Real-time PCR analysis for relative gene copy number quantification.

\( A \), real-time PCR efficiency for the MYC gene was determined with eight dilutions of COLO320DM DNA (0.39–50 ng) with three PCR repeats each. The melting curve \( B \) demonstrates the high purity of the PCR product. \( C \), CP cycles plotted vs log of input DNA quantities to calculate the slope. The real-time PCR method is sufficiently sensitive to distinguish one copy from two as shown for the androgen receptor (AR) gene in DNA samples from healthy males and females \( D \). The autosomal gene TOP2B was analyzed as a control \( E \). For each DNA sample, PCR was run in duplicate.
to 8.46 copies/cell). Only three cases for which CGH detected a gain at 8q had a MYC gain according to PCR. PCR detected a decrease in MYC gene copy number in six tumors, one of which also showed loss of genetic material at 8q24 by CGH.

The ratio of NNX3.1 to MYC was below the TI in 22 cases (63%), comprising distinct classes of genetic alterations: loss of NNX3.1 and normal MYC (8 tumors), normal NNX3.1 and gain of MYC (4 tumors), or loss of NNX3.1 concurrent with gain of MYC (7 tumors). CGH did not detect concurrent 8p loss and 8q gain in any of the seven tumors. Conversely, two tumors with concurrent loss at 8p and gain at 8q according to CGH were not detected by PCR. Four tumors exhibited loss of both genes or gain of both genes, leading to a significant decrease in the ratio of NNX3.1 to MYC below normal in three cases. Our findings concur with recent observations of an association between loss of genetic material at 8p and gain at 8q in prostate cancer (15, 16).

In summary, our relative real-time PCR quantification method based on the LightCycler system differs from previous methods in that it uses the fluorescent dye SYBR Green I, which rapidly and sensitively detects low amounts of DNA molecules. Compared with hybridization probes, SYBR Green I is less expensive, which becomes particularly important when screening of larger series of tissue samples is intended. As a modification of the $2^{-\Delta\Delta CT}$ method, we generated internal calibration curves to determine the gene-specific PCR efficiencies for each individual run. This approach allows for screening analysis of individual genes in separate runs under optimum PCR conditions. As demonstrated for prostate cancer samples, our real-time PCR quantification method is suitable for screening of specific genes and could be suitable for other applications in which gene copy number alterations are an important feature of pathogenesis.

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References


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Treatment of Maternal Blood Samples with Formaldehyde Does Not Alter the Proportion of Circulatory Fetal Nucleic Acids (DNA and mRNA) in Maternal Plasma, Satheesh Kumar Reddy Chinnappagari, Wolfgang Holzgreve, Olav Lapaire, Bernhard Zimmermann, and Sinuhe Hahn* (University Women’s Hospital/Department of Research, Basel, Switzerland; * address correspondence to this author at: Laboratory for Prenatal Medicine, University Women’s Hospital/Department of Research, Spitalstrasse 21, CH 4031 Basel, Switzerland; fax 41-61-265-9399, e-mail shahn@uhbs.ch)

Cell-free fetal DNA and fetal mRNA can be found in maternal plasma and used for noninvasive prenatal diagnosis and, potentially, for monitoring and prognosis of certain pregnancy-related clinical conditions (1–12). The excess of maternal DNA in these samples, however, complicates the detection of fetal genetic traits that are similar to those in the maternal genome (e.g., point mutations) (1, 13, 14). In normal pregnancies, fetal DNA represents only ~3–6% of the total DNA in maternal plasma (15). Thus, technical challenges lie in either developing methods permitting the reliable differentiation of fetal genetic loci or in reducing the amount of circulatory maternal DNA.

Dhallan et al. (16) have recently reported that the addition of formaldehyde to maternal blood samples increases the proportion of cell-free fetal DNA in maternal plasma by decreasing the concentration of maternal DNA. This effect was proposed to reflect an ability of formalde-