Genomic and Expression Analysis of the 3q25-q26 Amplification Unit Reveals TLOC1/SEC62 as a Probable Target Gene in Prostate Cancer

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Abstract
Gain at chromosome 3q25-q26 has been reported to commonly occur in prostate cancer. To map the 3q25-q26 amplification unit and to identify the candidate genes of amplification, we did fluorescence in situ hybridization and quantitative real-time PCR for gene copy number and mRNA expression measurements in prostate cancer cell lines and prostate cancer samples from radical prostatectomy specimens. The minimal overlapping region of DNA copy number gains in the cell lines could be narrowed down to 700 kb at 3q26.2. Of all positional and functional candidates in this region, the gene TLOC1/SEC62 revealed the highest frequency (50%) of copy number gains in the prostate cancer samples and was found to be up-regulated at the mRNA level in all samples analyzed. TLOC1/Sec62 protein was also shown to be overexpressed by Western blot analysis. Intriguingly, the TLOC1/SEC62 gene copy number was increased in prostate tumors from patients who had a lower risk of and a longer time to progression following radical prostatectomy. These findings make TLOC1/SEC62 the best candidate within the 3q amplification unit in prostate cancer. TLOC1/Sec62 protein is a component of the endoplasmic reticulum protein translocation machinery, whose function during prostate carcinogenesis remains to be determined.

Introduction
Gene copy number gain is a common mechanism by which genes achieve overexpression in tumors. For prostate cancer, amplifications of specific genes, such as c-MYC, HER2/neu, and androgen receptor, have been shown to be of prognostic relevance (1). However, most of the chromosomal regions found amplified in prostate cancer by comparative genomic hybridization (CGH) do not harbor known oncogenes. Although the amplification target genes remain mostly unknown, the presence of DNA copy number gains at particular regions may be associated with tumor aggressiveness or clinical outcome as shown for chromosome 7q (2, 3).

Most recently, we reported DNA copy number gains at chromosome 3q25-q26 in 50% of prostate carcinomas using CGH (4). Similar frequencies of 3q gains were described by others, although various sites on 3q are likely to be involved (5, 6). In this report, we describe the fine mapping of the 3q25-q26 amplification unit in prostate cancer and the identification of TLOC1/SEC62 as the gene with the highest frequency of gene copy number gains compared with other positional candidates. Overexpression at the mRNA and protein levels indicates a biological role of TLOC1/SEC62 in prostate cancer development.

Results
DNA Copy Number Gains on 3q25-q26 Are Frequent in Prostate Cancer as Detected by CGH
To assess frequency and extent of genetic alterations on chromosome 3q in prostate cancer, CGH was done on 3 prostate cancer cell lines and 22 primary prostate cancer samples. The CGH findings in the cell lines have been published elsewhere (7). In brief, DNA copy number gains on 3q had been detected in DU145 and DU145M1 encompassing the region 3q22-qter, whereas PC3 had displayed no copy number changes on 3q. Of the 22 primary tumor samples, 3q gains were documented in 8 (36.4%) cases with the minimal overlapping region mapping to 3q21-q26. Including our previous CGH series with a total of 80 prostate carcinomas (4, 8), the common region of DNA copy number gains can be narrowed down to 3q25-q26. The overall incidence of 3q gains involving 3q25-q26 in prostate cancer attains 31.3%.

Detailed Mapping of the 3q25-q26 Amplicon
We first did fluorescence in situ hybridization on the cell lines DU145, DU145M1, and PC3 applying 16 BACs within 3q25-q28 (Fig. 1). With this approach, the region with...
the highest signal numbers in the three cell lines could be narrowed down to 1.4 Mb within 3q26.2. This region was then analyzed in more detail using seven overlapping BACs (RP11-627P8, RP11-82C9, RP11-816J6, RP11-362K14, RP11-379K17, RP11-81O18, and RP11-543D10). The region between RP11-82C9 and RP11-816J6 encompassing 500 kb does not contain genes according to the Ensembl database (http://www.ensembl.org) and was therefore not investigated. With this step, the region with highest signal numbers could further be narrowed down to 700 kb between RP11-816J6 and RP11-543D10 at 3q26.2. It is of note that the highest signal numbers for these distinct BACs were detected in DU145 and DU145MN1, whereas PC3 showed only a slight increase above ploidy level.

Assessment of Candidate Genes around the 3q25-q26 Amplicon

Quantitative real-time PCR was done on 9 genes that were selected for analysis: (a) by having been assumed as functional candidates being located within the minimal region with the highest BAC signal numbers (MDS1, hTERC, SAMD7, and TLOC1/SEC62) or (b) by having been reported to be involved in tumorigenesis in other organs with a location of maximally 10 Mb upstream and downstream of the minimally amplified region [IL12A (4), SKIL (9), SLC2A2 (10), PIK3CA (11), and SOX2 (12)]. We first did a screening analysis in the cell lines PC3, DU145, and DU145MN1. For all genes, except SOX2, a copy number gain was detected in at least one of the three cell lines. Then, gene copy numbers of each gene, except SOX2,
were measured in the 22 primary prostate cancer samples (Fig. 2). TLOC1/SEC62 was the gene with the most frequent copy number gains found in 11 (50%) samples. TLOC1/SEC62 also revealed the highest gene copy number increase in the tumor tissues, which varied between 2.91 and 4.89 gene copies per cell. The copy number of SKIL was not found to be increased in any of the tumors. Due to lack of material, DNA from only a few tumors was available for the analysis of MDS1 and IL12A. In these, no copy number gain could be documented for either gene.

**Expression Analyses of Genes around the 3q25-q26 Amplicon by Quantitative Real-time Reverse Transcription-PCR**

Expression analysis was done using quantitative reverse transcription-PCR on the cell lines PC3, DU145, and DU145MN1 and on a subset of 13 prostate cancer samples. Only genes with documented copy number gains in prostate cancer tissues were included (hTERC, SAMD7, TLOC1/SEC62, SLC2A2, and PIK3CA). All selected genes were found to be upregulated in the cell lines compared with pooled normal prostate mRNA. In the prostate cancer samples, a >3-fold up-regulation was detected for the gene TLOC1/SEC62 in all 13 samples analyzed varying between 3.1- and 14.2-fold. Of these, 7 samples revealed significant gain of TLOC1/SEC62 gene copies. The genes SLC2A2, PIK3CA, and hTERC were found up-regulated >3-fold in 8, 7, and 5 samples, respectively. SAMD7 was not found to be expressed at all in the cancer samples.

As the most interesting candidate, TLOC1/SEC62 gene expression was further tested in 6 benign prostatic hyperplasia samples, which revealed a >3-fold up-regulation in only 2 samples (3.9- and 4.6-fold). The remainder varied between 0.69- and 2.85-fold.

**Expression Analyses of the TLOC1/SEC62 Gene by Western Blot Analyses**

To confirm the PCR data at the protein level and to address the question of functionality of the quantified mRNA, Western blot analysis was done. Similar amounts of protein that were derived from various cell lines were subjected to SDS-PAGE and Western blot analysis, employing an antipeptide antibody directed against the COOH terminus of TLOC1/Sec62 protein. In addition, the blots were analyzed with antibodies that were directed against Sec61
tub, an unrelated, ubiquitously expressed protein of the endoplasmic reticulum, and β-tubulin, a cytoskeleton-associated protein. The primary antibodies were visualized by luminescence imaging (Fig. 3). The ratio of TLOC1/Sec62 protein to β-tubulin and Sec61
tub, respectively, was determined for the benign prostate hyperplasia cell line BPHI (DSMZ no. ACC 143, German Resource Centre for Biological Material, Braunschweig, Germany) as well as for the prostate cancer cell lines PC3, DU145, and DU145MN1 in six independent analyses. Then, each ratio for the BPHI cells was independently set to 1 and the ratios for the cancer cell lines that were analyzed on the same blot were normalized to the BPHI ratios. On average, there was an ~1.5-fold increase in the ratios between TLOC/Sec62 protein and the control proteins in the case of the PC3 cells, whereas in the other two cancer cell lines the increase was ~3.5-fold. Thus, increased synthesis of TLOC1/Sec62 protein was observed in all tested tumor cell lines albeit to varying degree.

**Correlation of TLOC1/SEC62 Gene Copy Number Gain and Clinicopathologic Variables**

No association could be found between TLOC1/SEC62 gene copy number gain in the prostate cancer samples as determined by quantitative real-time PCR and Gleason score, stage, lymph node metastasis, or preoperative serum prostate-specific antigen. Clinical outcome was available from all 22 patients with a median follow-up time of 26 months (range, 5-82 months). Seven patients (6 patients with pT3 tumors and 1 patient with a pT1 tumor) showed progression. The median follow-up time of the 15 nonprogressors (10 patients with pT3 tumors and 5 patients with pT2 tumors) was 34 months (range, 15-82 months). Statistical analysis revealed a significant increase of the gene copy number gain in the prostate cancer samples as determined by quantitative real-time PCR and Gleason score, stage, lymph node metastasis, or preoperative serum prostate-specific antigen.

**FIGURE 2.** DNA copy number quantification of selected genes in 22 prostate cancer samples using real-time PCR. The genes are located within and around the area with the highest BAC signal numbers in the prostate cancer cell lines.
Discussion

Chromosome 3q gain has been identified in various types of tumors, including prostate cancer. Using CGH, we detected a common amplification unit encompassing 3q25-q26 in 31% of the prostate carcinomas analyzed. This is a rather high frequency similar to that of known amplification events involving MYC at 8q or the androgen receptor gene at Xq in this tumor type (1). Frequent involvement of chromosome 3q in prostate cancer was also described by Cher et al. (5), who discovered gains at 3q in 45% of androgen-independent prostate carcinomas and untreated metastases. A similar high frequency was reported in a more recent CGH study, although the minimal region was assigned to a more centromeric region at 3q (6). It is worth noting that two microarray-based CGH studies likewise confirmed involvement of 3q in prostate cancer, with gains at 3q13.33-q21.1 in the PC3 cell line (13) and, in accordance to our findings, at 3q26.2-q26.32 in primary prostate tumors (14).

Using fluorescence in situ hybridization, we could narrow down the minimally amplified region in the prostate cancer cell lines to a locus encompassing 700 kb at 3q26.2. To test for putative target genes of amplification within and around this locus, quantitative real-time PCR was done revealing copy number gains for the genes IL12A, MDS1, hTERC, SAMD7, TLOC1/SEC62, SKIL, SLC2A2, or PIK3CA in at least one of the prostate cancer cell lines analyzed. Determination of the copy number status of these genes in 22 prostate cancer samples revealed the gene TLOC1/SEC62 to be most frequently increased in 50% of the samples. Less frequently increased were the genes SAMD7 and SLC2A2 in 32% and 27%, respectively, and the genes hTERC and PIK3CA in 9% each. There were no SKIL gene copy number gains detected. Fitting these findings, the mRNA overexpression frequency was highest for TLOC1/SEC62 (100% of the prostate cancer samples) followed by SLC2A2 (62%) PIK3CA (54%), and hTERC (39%). SAMD7 was not found to be expressed at all. As TLOC1/SEC62 mRNA overexpression was not restricted to tumors with TLOC1/SEC62 gene copy number gain, other regulatory mechanisms have to be assumed emphasizing a general importance of this gene in prostate cancer development or growth. This assumption is in accord to other findings showing a generalized overexpression of genes, such as E2F3, in bladder cancer but with a gene copy number increase in only a subset of tumors (15).

Considering potential clinical implications, the TLOC1/SEC62 gene copy number turned out to be significantly increased in tumors from patients who did not develop progression following radical prostatectomy compared with those who did. Splitting the patients into two subgroups defined by the presence or absence of TLOC1/SEC62 gene copy

FIGURE 3. SDS-PAGE and Western blot analysis of prostate cancer (DU145MN1, DU145, and PC3) and benign prostate hyperplasia (BPH1) cell lines. Cytoskeleton-associated (α-tubulin) and endoplasmic reticulum–resident (Sec61p) proteins were quantified with respect to the Sec62p content. A single experiment is depicted as original together with its luminescence values (divided by 1,000). In addition, averaged and normalized values are shown as diagram (n = 6). The ratios of the detected proteins for BPH1 cells were set to 1.

FIGURE 4. Box plot showing the relation between TLOC1/SEC62 gene copy number in the tumor samples and clinical/biochemical progression after radical prostatectomy in 22 prostate cancer patients (15 nonprogressors and 7 progressors; P = 0.024). Dotted lines, cutoff values for significant TLOC1/SEC2 gene copy number deviation from normal.

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number gains, log-rank analysis revealed a lower risk of and a longer time to progression for patients with a TLOC1/SEC62 gene copy number gain. However, because of the small number of patients and the short follow-up time, precaution should be used in the interpretation of these results until larger studies and longer follow-up intervals have confirmed these data.

The high frequency of gene copy number gain and mRNA overexpression makes the gene TLOC1/SEC62 the best candidate within the 3q25-26 amplification unit in prostate cancer. This assumption was strongly supported by Western blot analyses, which showed an up to 3.5-fold increase of TLOC1/SEC62 protein production in the DU145 and PC3, for which no gene copy number gain could be documented but that showed increased mRNA content, was increased by a factor of 1.5 compared with benign prostate hyperplasia cell line BPH1.

TLOC1/SEC62 protein is a component of the endoplasmic reticulum translocation machinery. First described in the yeast Saccharomyces cerevisiae, the protein transport across the endoplasmic reticulum membrane is mediated by a membrane protein complex that consists of the trimeric Sec61p complex and the Sec62p-Sec63p subcomplex (16, 17). Although the association of TLOC1/SEC62 protein with the Sec61 complex in mammalian cells indicates involvement of this protein in transport processes similar to those done by the yeast Sec61 complex (18, 19), the precise function of the TLOC1/SEC62 protein in mammalian cells is still unknown. Our finding of TLOC1/SEC62 overexpression in prostate cancer cells is the first description of TLOC1/SEC62 up-regulation in tumor cells at all. Additional work will be required to precisely define its pathogenetic role, if any, in prostate cancer development. It is noteworthy that the orthologue of TLOC1/SEC62 in Drosophila, termed DTRP1, is maximally expressed at certain stages of embryonic and pupal development and that production of one of the two DTRP1 transcripts is by far the highest in the paragonial glands, which are somewhat equivalent organs to prostate glands (20). Furthermore, we note that TLOC1/SEC62 protein is classified as a potential nonselective cation channel by the protein families database of alignments and that another nonselective cation channel, TRPV6, is overexpressed in human prostate cancer cells (21).

In addition to prostate cancer, gain of sequences at 3q has also been identified in other malignancies, including tumors of the cervix (22), ovary (23), lung (24), and esophagus (9). Although the region 3q26 seems to be consistently involved, the molecular counterpart seems less consistent in the different tumor types. Whereas SKIL is a likely target in esophageal carcinoma (9), PIK3C4 and hTERC seem to play a biological role in cancer of the ovary and cervix, respectively (11, 22). We could document gene copy number gain and overexpression of the gene TLOC1/SEC62 in prostate cancer but could exclude SKIL and PIK3C4 as amplification targets in this tumor type. It seems most likely that the 3q26 region harbors various genes that contribute differentially to carcinogenesis in different organs. The biological role of TLOC1/SEC62 in prostate carcinogenesis and the value of TLOC1/SEC62 gene copy number gain as a diagnostic or prognostic marker in prostate cancer remain to be clarified in future studies.

Materials and Methods

Cell Lines and Tissue Samples of Prostate Carcinoma

For fine mapping of the 3q25-q26 amplification unit, prostate cancer cell lines DU145 (ATCC no. HTB-81) and PC3 (ATCC no. CRL-1435) as well as the derived subline DU145MNI (7) were used. Quantitative gene copy number and expression measurements of positional candidates were done on primary prostate adenocarcinoma samples, which were obtained after radical prostatectomy from thus far untreated prostate cancer patients. The specimens represent a subset of a previously

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NOTE: Cycling conditions: initial activation, 95 °C for 15 minutes; template activation for 50 cycles with denaturation at 94 °C for 15 seconds, primer annealing at T_m for 20 seconds, and extension at 72 °C for 20 seconds. Melting curve analysis: after 50 cycles 10 °C for 15 seconds and increase temperature up to 99 °C with a heating rate of 0.1 °C/s and a continuous fluorescence measurement.
described series (25) that was selected by the criteria of a complete standardized patient follow-up. Following prostatectomy, the specimens were dissected by a pathologist, snap frozen, and stored at −80°C. Only samples containing >50% tumor cells were included in the study. In the present subset of 22 prostate cancer samples, 6 carcinomas were staged as pT2 and 16 as pT3 according to the tumor-node-metastasis classification system, 5th revision (26). Concerning Gleason sum, 2 were graded as <7, 11 as 7, and 9 as >7. Lymph node metastases were present in 6 cases. All patients were clinically staged as M0. Following surgery, clinical status and serum prostate-specific antigens were monitored every 3 months during the first year, every 6 months in the second year, followed by yearly monitoring. The end point for calculating the survival time was defined by time from radical prostatectomy to either biochemical (prostate-specific antigen >0.2 ng/mL or rising prostate-specific antigen in three consecutive measurements) or clinical progression. The study was approved by the local ethics committee.

**Comparative Genomic Hybridization**

CGH was done as described (7). Applying nick translation, 1 μg test DNA was labeled with biotin-16-dUTP and control DNA with digoxigenin-11-dUTP. Biotinylated DNA was visualized by the local ethics committee.

**Fluorescence in situ Hybridization**

BAC clones from the human RPCI-11 and RPCI-13 BAC libraries were hybridized to interphase nuclei and metaphase spreads of the cell lines (Fig. 1). Physical locations of BAC clones were based on the information available at the University of California at Santa Cruz Human Genome Browser Gateway (http://genome.ucsc.edu; RP11-286N6, RP11-91L9, RP11-209H12, RP11-67F24, RP11-90M7, RP11-190F16, RP11-148D23, RP11-292L5, RP11-245C23, RP11-259I19, RP11-102G2, RP11-12L14, RP11-125E8, and RP11-63M3, and RP11-79K10) and the Ensembl database (http://www.ensembl.org; RP11-627P8, RP11-82C9, RP11-816J6, RP11-362K14, RP11-379K17, RP11-8108, and RP11-543D10). The clones were obtained from BACPAC Resource Center Children’s Hospital (Oakland Research Institute, Oakland, CA). Bacterial cultures and DNA isolation were done according to the BACPAC Miniprep protocol (http://www.biologia.uniba.it/rmc). Alu-PCR products of the BACs were used as probes and were biotinylated using nick translation. A digoxigenin-labeled D3Z1 probe specific for the centromeric region of chromosome 3 (Qbiogene, Illkirch, France) served as internal control. Dual-color fluorescence in situ hybridization and detection of fluorescence signals were done as described previously (7) with the following modifications: the hybridization mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, 10 μg/mL human Cot-1 DNA, 500 ng BAC probe DNA, and 0.5 µL D3Z1 probe DNA. This mixture was denatured at 75°C for 5 minutes and preannealed in a 37°C water bath for 15 minutes before it was applied to the slides.

**Quantitative Real-time PCR**

Quantitative real-time PCR for gene copy number measurement was done as described recently (27) using the LightCycler system (Roche, Mannheim, Germany) and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). Relative gene copy number was calculated from the real-time PCR efficiencies (E), which were determined for each individual run, and the crossing point (CP) deviations of the target and reference genes in a test sample versus a control following the equation: 

\[ \frac{E_{target} - E_{control}}{E_{reference} - E_{control}} \]

Because the gene copy number is 2 in normal diploid DNA, the relative copy number was multiplied by 2 to achieve the copy number in the test sample for the target gene. PCR was done at least in duplicates with 20 μL reaction volumes containing 10 μL of 2× QuantiTect SYBR Green PCR Master Mix, 0.5 μmol/L of each primer, 2.5 to 5 mmol/L MgCl₂, and 25 ng genomic DNA. Primer sequences and cycling variables are listed in Table 1. GAPDH, which is located at 12p13, served as reference gene. The region 12p13 has been shown not to be involved in prostate cancer. The existence of specific primer binding sites in pseudogenes of GAPDH was excluded by use of the BLASTN algorithm.

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NOTE: Cycling conditions: initial activation, 95°C for 15 minutes; template activation for 50 cycles with denaturation at 94°C for 15 seconds, primer annealing at Tm, for 20 seconds, and extension at 72°C for 20 seconds. Melting curve analysis: after 50 cycles 70°C for 15 seconds and increase temperature up to 99°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement.
To determine whether real-time PCR quantification on individual test samples was significantly different from the mean of measurements made on samples from normal individuals, the mean ± 2 × SD copy number values for each gene analyzed were determined in a total of 8 normal individuals: *IL12A* (2.16 ± 0.52), *MDM1* (2.19 ± 0.38), *hTERC* (1.88 ± 0.54), *SAMD7* (1.94 ± 0.42), *TLOC1/SEC62* (2.00 ± 0.60), *SKIL* (2.50 ± 0.48), *SLC2A2* (1.92 ± 0.24), *PIK3CA* (2.08 ± 0.28), and *SOX2* (1.88 ± 0.64).

**Quantitative Real-time Reverse Transcription-PCR**

Quantitative real-time PCR for mRNA expression was done using the LightCycler system and the QuantiTect SYBR Green PCR kit. Total RNA was isolated using the RNeasy Mini kit (Qiagen). RNA quality was controlled by spectrophotometrical quantification and agarose gel electrophoresis. Reverse transcription of 1 μg total RNA was done with SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) using oligo(dT)12–18 primer (Invitrogen) and RNase OUT (Invitrogen) after pretreatment with RNase-free DNase I (Stratagene, Amsterdam, the Netherlands). PCR was done at least in duplicates with 20 μL reaction volumes containing 10 μL of 2× QuantiTect SYBR Green PCR Master Mix, 0.5 μmol/L of each primer, and 2 μL cDNA (1:20 dilution). Primer sequences and cycling variables are listed in Table 2.

Real-time PCR efficiencies were determined by creating external standard curves with cDNA from DU145MN1. Of three housekeeping genes tested, *B2M* was chosen as reference gene. Relative quantification was calculated with the Relative Quantification Software version 1.01 (Roche). DU145MN1 cDNA was measured in each run and served as calibrator to correct for run-to-run variations. Final results are expressed as n-fold differences in target gene expression in cancer samples relative to normal prostate.

**Western Blot Analysis**

The rabbit antibodies were directed against the COOH-terminal peptide of TLOC1/Sec62 protein (CGETPKSSHEKS), COOH-terminal peptide of Sec61apo (CKEQEVGSM-GALLF), and β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies reacted only with the expected proteins in immunoblots. The samples were analyzed by electrophoresis in 12.5% polyacrylamide gels and subsequent electroblotting to polyvinylidene fluoride membranes followed by incubation with specific antibodies and peroxidase conjugate of goat anti-rabbit IgG. The antibodies were visualized by incubation of the blots with enhanced chemiluminescence (Amersham Biosciences, Freiburg, Germany) and subsequent analysis with a Lumi-Imager F1 (Roche).

**Statistical Analysis**

For all variables complying with normal Gaussian distribution, Student’s *t* test was applied for comparison of independent sample sets. Variables not complying with normal Gaussian distribution were analyzed by Mann-Whitney *U* test. All *P* values were based on two-sided tests and the threshold to accept statistical significance was set at the *x* level 0.05. Log-rank test was applied as statistical hypothesis test procedure for the comparison of time to progression after surgical therapy in the independent groups of tumors with a *TLOC1/SEC62* gene copy number gain and those without. Analyses were done with the Statistical Software Package SPSS version 10.0 (SPSS, Chicago, IL).

**References**


Molecular Cancer Research

Genomic and Expression Analysis of the 3q25-q26 Amplification Unit Reveals *TLOC1/SEC62* as a Probable Target Gene in Prostate Cancer

Volker Jung, Roland Kindich, Jörn Kamradt, et al.


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