Overexpression and Mislocalization of the Chromosomal Segregation Protein Separase in Multiple Human Cancers

Rene Meyer, Viacheslav Fofanov, Anil K. Panigrahi, Fatima Merchant, Nenggang Zhang, and Debananda Pati

Abstract

Purpose: Separase, an endopeptidase, plays a pivotal role in chromosomal segregation by separating sister chromatids during the metaphase to anaphase transition. Using a mouse mammary tumor model we have recently shown that overexpression of Separase induces aneuploidy and tumorigenesis (Zhang et al., Proc Natl Acad Sci 2008;105:13033). In the present study, we have investigated the expression level of Separase across a wide range of human tumors.

Experimental Design: To examine the expression levels and localization of Separase in human tumors, we have performed immunofluorescence microscopy using human Separase antibody and tumor tissue arrays from osteosarcoma, colorectal, breast, and prostate cancers with appropriate normal controls.

Results: We show that Separase is significantly overexpressed in osteosarcoma, breast, and prostate tumor specimens. There is a strong correlation of tumor status with the localization of Separase into the nucleus throughout all stages of the cell cycle. Unlike the normal control tissues, where Separase localization is exclusively cytoplasmic in nondividing cells, human tumor samples show significantly higher number of resting cells with a strong nuclear Separase staining. Additionally, overexpression of Separase transcript strongly correlates with high incidence of relapse, metastasis, and lower 5-year overall survival rate in breast and prostate cancer patients.

Conclusion: These results further strengthen our hypothesis that Separase might be an oncogene, whose overexpression induces tumorigenesis, and indicates that Separase overexpression and aberrant nuclear localization are common in many tumor types and may predict outcome in some human cancers.

An evolutionarily conserved protein complex called cohesin holds sister chromatids together to allow accurate separation of sister chromatids into two daughter cells. At the onset of anaphase, Separase, an endopeptidase, is activated and cleaves the cohesin subunit Rad21 (also called SCC1 or MCD1), which releases sister chromatin cohesion. Separase activity is tightly regulated via several mechanisms (for details, see refs. 1–3) to ensure accurate and precise activation of cohesin Rad21 cleavage during the metaphase to anaphase transition (2–4).

Separase is activated after its inhibitory chaperone securin is degraded by APC-mediated phosphorylation and ubiquitin-mediated degradation (1, 5–8). Additionally, phosphorylation of Separase on Ser1126 and Thr1326 residues is a second mechanism to inhibit Separase activity (9, 10). Therefore, Securin null cells are viable and appear to have a nearly normal cell cycle (11–13). However, premature separation of sister chromatids, for example, by premature activation of Separase or by insufficient inhibition of overexpressed Separase, is thought to result in aneuploidy (14).

Knockout of the Separase gene results in embryonic lethality in mice (13, 15). Small interfering RNA-mediated knockdown of Separase results in genomic instability (8, 16), also seen in Separase-deficient mouse embryonic fibroblasts. No severe haploinsufficiency in Separase heterozygous mouse has been observed,4 suggesting that moderately lower level of Separase is sufficient for normal cell cycle progression in vivo. On the contrary, there are several lines of evidence that overexpression of Separase can lead to premature sister chromatid separation, anaphase bridges, and lagging chromosomes (17, 18).

Aneuploidy is a hallmark of human cancers (19) and is especially high in osteosarcoma, breast, and prostate cancers (20, 21). Although there have been many proposed hypotheses, there is no general agreement as to why aneuploidy is so highly...
For each of the 10 tumors and 5 normal tissue specimens obtained from the M. D. Anderson Cancer Center repository, tissue was embedded in OCT (Sakura Tissue-Tek) medium and refrozen at -20°C before cryosectioning and immunofluorescence staining.

**Separase overexpressed cell lines.** Human Separase was overexpressed constitutively in human cervical cancer cell line HeLa and conditionally in the diploid, nonmutogenic FSK3 mammary epithelial cells. In brief, HeLa cells were transfected with a cytomegalovirus-driven, neomycin-resistant HA-tagged-hSeparase plasmid and selected for stable integration by treating the cells in the presence of G418 (geneticin; Invitrogen). Empty vector-transfected clones served as controls. The hSeparase protein expression of transfectants was detected by Western blot analysis using HA-epitope antibody and subsequently verified with commercially available Separase antisera (Abnova). The clone selection was based on the expression level of Separase protein compared with the empty vector controls. Details of the Tet-inducible FSK3 Separase cells have been described previously (18).

**Immunofluorescence microscopy.** Human tissue arrays and paraffin-embedded samples were baked for 2 h at 60°C and deparaffinized in xylene for 2 × 10 min, respectively. Following stepwise rehydration in 100%, 95%, 80%, 75%, and 30% ethanol for 10 min each and 2 × 5 min in deionized water, antigen retrieval was done in a pressure cooker at 121°C for 20 min in buffer (14.5 mL of 0.1 M citric acid monohydrate + 61.5 mL of 0.1 M sodium citrate in 750 mL double-distilled H2O at pH 6.0). Nonspecific binding was blocked with 10% normal goat serum in PBS at room temperature for 3 h before incubation with primary anti-Separase antibody (Abnova ESPL1-6H6) and normal mouse IgG as control antibody overnight at 4°C in 10% normal goat serum/PBS in a humidified chamber. After rinsing with PBS on a shaker for 4 × 15 min, incubation with secondary antibody (goat anti-mouse IgG-rhodamine conjugate) was done in 10% normal goat serum/PBS in a humidified chamber at room temperature for 1.5 h. To detect the proliferation status of the cells in the tissue array, a set of slides were counterstained with Ki-67 antibody (Abcam) followed by a secondary goat anti-rabbit FITC antibody (Molecular Probes) for detection. After PBS rinse on a shaker 3 × 10 min, slides were mounted using Vectashield mount medium with 4,6-diamidino-2-phenylindole and kept at 4°C if not immediately imaged on a Nikon eclipse E800 microscope. The images were processed using background subtraction to remove shading due to nonuniform illumination and inhomogeneous staining effects and using color compensation to minimize the effects of spectral bleed-through among the three-color channels (red, green, and blue). The algorithms are described in detail elsewhere (25, 26).

**Statistical analysis.** For each cancer subtype, the associations of the localization of Separase expression (nuclear versus cytoplasm), the magnitude of the expression signal, and the disease status (tumor versus normal) were modeled using a logistic regression framework. Forward selection model construction approach (27), in which covariates are incorporated into the regression model in the order of statistical significance, was used to construct the final logistic regression model and assess the strength of association between each of the considered covariates and disease status. ANOVA framework was used to establish the statistical significance for the associations between covariates and disease status.

The protein expression levels of Separase in human breast tumor specimens were compared statistically with the matched normal tissues using a set of paired tests including paired t test, rank-sum test, and signed rank test. Rank-sum and signed rank tests are more robust to departures from normality and do not have restrictive assumptions. The immunofluorescence staining data for Separase expression in these matched normal versus tumor samples and the matched controls versus disease from the tissue arrays were used for the paired t test analysis. For the purposes of statistical analysis, the localization of Separase expression was separated into distinct binary variables, indicating absence/presence of Separase in the nucleus and absence/presence of Separase in the cytoplasm. For each sample, the average expression levels and
localization in the 100 cells evaluated in each of the three randomly selected microscopy spots were calculated. The magnitude of expression, evaluated using either propensity and intensity scores or using the combined total score, was also included in the statistical model (28, 29). Due to the high degree of correlation between propensity and intensity scores in the observed data, combining the two into the total score did not have a significant effect in the inferences drawn from the logistic regression model.

Results

To examine the expression levels and localization of Separase in human tumors, we have performed immunofluorescence microscopy using human Separase antibody and tumor tissue arrays from osteosarcoma, colorectal, breast, and prostate cancers with appropriate normal controls. Separase expression was scored according to standard pathology scoring with a propensity score ranging from 0 to 5 and an intensity score from 0 to 3 (28, 29). The combined addition of propensity score and intensity score was used as total score for logistic regression analysis in a stepwise forward model (27). Furthermore, we also scored the expression depending on Separase localization to cytoplasm, nucleus, or both compartments. Table 1 summarizes the data set specifications with total number of cases and controls that were used for the analysis passing tissue quality-control and scoring confidence. For a summary of the raw scoring data, see Supplementary Table S1A-C. We first looked at the tumor types in different organs individually and where possible used a paired t test for tumor and matched normal control tissues from the same patient.

Breast cancer. The paired t test for ductal carcinomas where we had matched normal tissue from the same patient (tissue repository) shows a highly significant correlation of high Separase expression in tumors for the propensity score ($t = -4.36; \, P = 0.0024$) and an even higher correlation for the intensity score ($t = -4.91; \, P = 0.0012$). Figure 1 shows representative samples of Separase staining in normal and breast cancer samples. Using a logistic regression model with forward selection on all normal versus all breast tumors (tissue repository samples and arrays), the factor of nuclear expression is found to be the most significant one with $t = 3.4228$ and $P = 0.00075$. Hence, overexpression of Separase in the nucleus is the strongest positive contributing factor for tumors followed by intensity and propensity scores. In a full covariant model, the nuclear localization was the only statistically significant contributor at $P = 0.0020$. Propensity and intensity are most likely not independent contributors and hence did not reach significance in this model. However, if total score (propensity + intensity) is used, the full covariant model predicts high Separase expression as the strongest contributor for tumors at $t = 6.503 (P = 9.69 \times 10^{-15})$ and the nuclear localization as the second strongest at $t = 5.172 (P = 6.90 \times 10^{-7})$

Prostate. We analyzed a total of 58 samples including 9 normal controls, 37 hyperplasias, and 22 tumors. Because it is debatable if prostate tissue adjacent to a prostate adenocarcinoma actually is normal or not (30–32), we examined two types of control tissue including 3 samples from adjacent “normal” tissues and 6 samples from healthy prostates. The 3 adjacent normal tissue samples had slightly elevated Separase protein expression and a substantial amount of interphase cells with nuclear Separase localization. These findings were not seen in the 6 normal healthy controls. Therefore, the nuclear localization as predictor for prostate cancer did not reach significance with the 3 adjacent controls having nuclear Separase expression. If we exclude these 3 samples, the nuclear localization is again the strongest predictor of the malignant phenotype (Fig. 2).

Comparing the 22 prostate carcinomas with all 9 controls, the intensity of Separase expression is the strongest predictor for tumors at $t = 9.968 (P = 7.10 \times 10^{-15})$ followed by the propensity score at $t = 3.689 (P = 0.00092)$. Figure 2 shows representative samples of Separase staining in normal and prostate adenocarcinoma samples. We observed detectable levels of Separase expression in <5% of the normal prostate cells. If we assume a higher proliferation index in hyperplastic and tumor samples, this still does not account for the number of strong Separase-expressing cells, because we observed this in 30% to 100% of cells in different tumor specimens.

Based on linear discriminant analysis, 35 of the 37 hyperplastic samples were closer to tumor samples than normal controls ($P = 0.00243$ to $1.71 \times 10^{-6}$), implying that hyperplasia more closely resembles a stage closer to prostate cancer with regard to the high Separase expression and its nuclear localization. This is another indication that the increase in Separase expression is one of the early steps in malignant transformation.

Osteosarcoma and colorectal carcinoma. Analysis of 63 osteosarcoma specimens and 4 normal controls on the CHTN2003CRCprog array indicated that nuclear overexpression of Separase was the strongest predictor of tumor tissue with a $t = 10.506 (P = 2.66 \times 10^{-15})$. The intensity of Separase expression with $t = 3.262 (P = 0.0018)$ as well as the propensity score with $t = 3.006 (P = 0.0038)$ were also statistically significant contributors. Figure 3 shows two of the normal controls and two representative examples of osteosarcoma.

However, in colorectal cancers, Separase expression was found to be very high in both normal nonneoplastic and neoplastic colon. We did not detect any significant difference in either propensity or intensity scores between colon cancer subtypes including adenoma, metastatic cancer, and primary carcinoma. Nuclear localization also did not reach significance to distinguish tumor from normal colon.

Separase localization and proliferation. To address the question, if stronger Separase staining correlates merely with increased proliferation, we counterstained tissue array samples and cultured cells with the proliferation marker Ki-67 (green fluorescence) and Separase (red fluorescence). The majority of osteosarcoma and breast cancer samples on the tissue arrays

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Samples</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>121</td>
<td>103</td>
<td>18</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>59</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>Prostate</td>
<td>68</td>
<td>59</td>
<td>9</td>
</tr>
<tr>
<td>Colon</td>
<td>68</td>
<td>42</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>316</td>
<td>259</td>
<td>57</td>
</tr>
</tbody>
</table>

NOTE: Number of cases and controls that were included in the statistical analysis of Separase expression correlation with tumor status.
clearly show that high Separase expression is constitutively nuclear regardless of the proliferative status of the cells (Fig. 4). In contrast, normal controls have very low or undetectable Separase expression, except for the proliferating cells; hence, a comparison of cytoplasmic versus nuclear localization is not feasible. The only normal breast sample that has relatively high Separase expression in resting cells shows clear exclusion of the Separase from the nucleus in the majority of interphase cells (Fig. 4). We were unable to test the prostate tumor samples for Ki-67 counterstaining due to the unavailability of additional array. Additional studies using a stable Separase-overexpressing HeLa clone and a Tet-inducible diploid, nontumorigenic mouse mammary epithelial cell line indicated that Separase nuclear localization correlates with its overexpression irrespective of the proliferative status (see Supplementary Fig. S4). The induction of Separase expression in
the FSK cell lines clearly shows a shift of Separase localization from exclusively cytoplasmic in uninduced cells to an evenly cytoplasmic and nuclear staining after 3 days of doxycycline-induced Separase expression (Supplementary Fig. S4), suggesting that overexpression of Separase may contribute to its aberrant localization to nucleus.

**Summary.** For all the investigated tumor types, except colon carcinoma, not only high expression levels but also the aberrant nuclear localization of Separase was the strongest predictor of tumor status. Both propensity and intensity scores showed a significantly higher Separase protein expression in tumors compared with normal tissue controls. The correlation between Separase overexpression in tumor also holds good using total score. Table 2 summarizes the logistic regression data for all cancer types using a stepwise forward model. Our data clearly show very strong nuclear localization and increased expression of Separase in osteosarcoma, breast, and prostate tumors.

**Discussion**

We recently reported that induced overexpression of Separase in a mouse mammary model causes aneuploidy and tumor formation in vivo (18). Using Western blot analysis of a limited number of breast tumor specimens, we also showed that Separase protein is significantly overexpressed in human breast tumors compared with the matched normal controls (18). There are several published studies analyzing the mRNA expression levels from breast cancers versus normal controls and metastasis (21, 33–35). We reevaluated these data available on the Oncomine database for Separase mRNA expression levels with respect to tumor status and patient outcome data. Separase transcript level is highly correlated with tumor status (36–38). Separase mRNA levels are found to be consistently higher in tumors compared with normal controls at highly significant P values. None of these studies identified Separase as one of the top 70-gene (39) or 64-gene (34) signatures that would predict breast cancer patient outcome when measured in the primary tumor in these studies. Notably, there are only 3 genes in common between these two studies, indicating that the top 70 or 64 genes selected by the cutoff criteria might not be the most important genes but the most intensely altered expressions in these patient cohorts. It is interesting to note that high Separase expression in both studies correlates with a higher incidence of recurrence (t test, -7.415; \( P = 2.8 \times 10^{-11} \)) and lower 5-year survival rate (t test, -4.594; \( P = 1 \times 10^{-5} \); see Supplementary Fig. S1). Supplementary Fig. S2 shows the correlation between high Separase mRNA levels and breast tumors if compared with normal breast (35).

Based on our analysis of Separase mRNA expression data from different tumor grades in the Oncomine database, we also found a correlation between high Separase expression and high-grade tumors. Additionally, the Uppsala study (33) also negatively correlates Separase expression with disease-free and overall survival (t test, -4.413; \( P = 2 \times 10^{-5} \)), indicating that there might be a correlation in all breast cancer patients, although it is not one of the top 70 genes with the highest alteration in expression levels.

At least one published study (31) contains mRNA expression data for Separase in prostate cancers positively correlating it with tumor status. In metastatic prostate tumors, Separase expression is found to be particularly high with a correlation factor of 0.805 (\( P = 3.210^{-5} \); see Supplementary Fig. S3). These results further strengthen our observation that overexpression

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**Fig. 3.** Representative immunofluorescence pictures of normal bone (left) and osteosarcoma samples (right) at the indicated magnifications. Red, Separase expression; blue, DNA (4',6-diamidino-2-phenylindole staining). Bar, 150 μm (×20) and 30 μm (×100).
of Separase correlates with malignant transformation and should be evaluated as a novel biomarker for detection and prediction.

In our study, we have here shown that Separase is significantly overexpressed in multiple human solid tumors including breast, prostate, and osteosarcoma. Interestingly, it is not only the total cellular level of Separase protein but also its aberrant nuclear localization that constitutes the strongest statistical contributor to distinguish tumor from normal tissue. These cells with nuclear Separase localization represent the majority of the total tumor mass. The mechanistic significance of nuclear Separase localization is unclear, but there are several possible explanations. First, it is possible that the normal mechanism of active nuclear exclusion of Separase (40) may be overwhelmed by Separase overexpression. Second, export of Separase from the nucleus of proliferating tumor cells may be inefficient owing to Separase overexpression. Third, high Separase level and its nuclear localization may poise the cells for division. Finally, it is known that cohesin is recruited to damaged sites along chromosomes during repair, and it is removed by Separase following DNA repair (16, 41). Hence, nuclear retention of Separase in proliferating tumor cells could result in premature removal of cohesin, a process normally occurring only after the repair process is complete. Premature cohesion removal would enhance mutation defects in the tumor DNA-damage response.

Separase might be important for DNA damage repair (16). How overexpression and nuclear localization is connected or contributes to tumor formation/progression is not yet understood. In mouse mammary epithelia cells, transcriptional regulation of Separase expression is regulated by estrogen and progesterone, and Separase expression is further facilitated by loss of p53 (17). The observation that Separase overexpression and loss or mutation of p53 strongly correlate in breast cancers (33, 42) might not be coincidental. These findings strengthen the hypothesis that misregulation of sister chromatid cohesion and segregation and the resultant aneuploidy could be a strong driving force for tumorigenesis and/or tumor progression.

Recently, several studies have focused on characterizing tumor transcriptomes of gliomas (43), ovarian (44, 45), breast (21, 35, 39), bladder (46), and prostate cancers (31). While analyzing these published data sets, we found a strong positive correlation of Separase mRNA expression with tumor grade and a strong negative correlation with disease-free and overall survival. Furthermore, studies show that overexpression of

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**Fig. 4.** Immunofluorescence pictures of tissue array samples costained with Ki-67 (green) and Separase (red). Blue, DNA by 4',6-diamidino-2-phenylindole staining. **Rows 1 and 2,** two different types of breast cancer specimens (IDC, intraductal carcinoma; DC, ductal carcinoma); **row 3,** representative osteosarcoma sample; **row 4,** normal breast specimen with detectable Separase expression showing the majority of Separase excluded from the nucleus. The majority of normal breast samples have undetectable or very low Separase staining. Black and white single channel and the merged pictures are shown for better appreciation of Separase localization to cytoplasm and nucleus in the breast cancer and osteosarcoma sample. Bar, 25 μm.
Separase strongly correlates with mutations in p53 (33, 42) as well as BRCA1 (34) in breast cancer patients. These studies not only strengthen our hypothesis that Separase overexpression plays an important role in mammary carcinogenesis (17) but also suggest that it may be a more common feature of human malignancies. Aberrant nuclear localization of the Separase in the tumor may not be the sole contributing factor because the intensity score, propensity score, or combined total scores are also highly significant in the forward selection model, indicating that Separase protein expression strongly correlates with tumor progression. Therefore, we suggest that Separase expression analysis can be considered as a factor to predict metastasis and patient outcome for primary tumor analysis.

### Table 2. Summary of logistic regression analysis using stepwise forward model selection to identify parameters that predict disease status including all investigated samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Akaike information criterion</th>
<th>Model coefficients</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nu</td>
<td>33.15</td>
<td>0.129</td>
<td>0.00075</td>
</tr>
<tr>
<td>nu + TS</td>
<td>32.96</td>
<td>0.123 + 0.026</td>
<td>0.0018 + 0.075</td>
</tr>
<tr>
<td>Osteosarcoma status</td>
<td>-58.11</td>
<td>0.333</td>
<td>2.66 \times 10^{-15}</td>
</tr>
<tr>
<td>nu + TS</td>
<td>-69.34</td>
<td>0.283 + 0.036</td>
<td>1.15 \times 10^{-12} + 0.00040</td>
</tr>
<tr>
<td>nu + TS + cy</td>
<td>-72.28</td>
<td>0.254 + 0.048 - 0.048</td>
<td>2.73 \times 10^{-10} + 3.82 \times 10^{-8} + 0.0320</td>
</tr>
<tr>
<td>Prostate cancer status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td>5.38</td>
<td>0.191</td>
<td>1.53 \times 10^{-9}</td>
</tr>
<tr>
<td>TS + cy</td>
<td>-3.06</td>
<td>0.176 - 0.138</td>
<td>6.72 \times 10^{-10} + 0.00023</td>
</tr>
<tr>
<td>Combined all cancers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nu</td>
<td>61.85</td>
<td>0.196</td>
<td>1.89 \times 10^{-12}</td>
</tr>
<tr>
<td>nu + TS</td>
<td>30.93</td>
<td>0.154 + 0.063</td>
<td>2.07 \times 10^{-7} + 1.39 \times 10^{-8}</td>
</tr>
<tr>
<td>nu + TS + cy</td>
<td>27.12</td>
<td>0.132 + 0.071 - 0.068</td>
<td>6.9 \times 10^{-7} + 9.69 \times 10^{-10} + 0.0174</td>
</tr>
</tbody>
</table>

NOTE: Associations between disease status and expression of Separase: expression in the nucleus (nu), expression in cytoplasm (cy), and degree of expression measured by combining propensity and intensity scores (TS). Consistent with the stepwise forward model selection paradigm, only the statistically significant contributors (covariates) are included in the model. Lower Akaike information criterion values indicate a better predictive value. Statistical analysis was carried out using S-Plus software (TIBCO Software).

### References

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