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Caspase 3 as an alternative marker for increasing the throughput of the predictive radiation-induced lymphocyte apoptosis assay in malignancies

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More than half of all patients treated for their malignancy will receive radiotherapy. For breast cancer patients, the risk for moderate to severe late effects is reported to be around 30%. This will become of more relevance as the number of long-term survivors is increasing. Individualized radiotherapy may help to reduce the side effects of RT but will require identification of the patient's radiosensitivity before treatment. The radiation-induced lymphocyte apoptosis (RILA) assay attracts much attention as a simple and inexpensive predictive assay for clinical radiosensitivity. However, the drawback of the RILA assay is that the assay has relatively long assay time and therefore a relatively low throughput. The whole procedure of the standard RILA assay requires at least 72h, with a 24h pre-irradiation and 48h post-irradiation incubation time. In this study, a modification of the assay was developed and tested to demonstrate whether the test time can be shortened and thereby its throughput increased. In total, 37 patients enrolled in this study including 26 breast cancer and 11 prostate cancer patients. Using flow cytometry, we established and tested a modification of the RILA assay that detects caspase 3 activity that is an essential enzyme early in the apoptosis cascade, rather than detecting lymphocyte apoptosis by DNA degradation, which can be observed in late apoptosis.

According to the results from breast and prostate cancer patients (mixed malignancy cohort), the median value of PI RILA in $CD4^+$ cells was 7.6 % (range: 0.4 % - 32.5 %). As expected, the median value of PI RILA in $CD8^+$ cells were higher than in $CD4^+$ cells (median value: 25.4 %, range: 0.90 % - 61.30 %). As in the breast cancer patients, PI RILA had a median value of 8.0 % (range: 0 - 32.5 %) in $CD4^+$ T cells and 16.2 % (range: 0.9 % - 61.3 %) in $CD8^+$ T cells. Prostate cancer patient median values of PI RILA in $CD4^+$ T lymphocytes were 7.6 % (2.8 % - 13.1 %), while the median values of PI RILA in $CD8^+$ T lymphocytes were 31.6 % (10.5 % - 59.2 %).

In order to show if a caspase-based RILA assay correlates with the PI RILA assay in CD4 and $CD8^+$ lymphocytes, regression analysis was performed between the caspase 3 RILA and the PI RILA assay after 48h post irradiation. The caspase 3 RILA assay showed higher RILA values compared with those of the PI RILA assay for both $CD4^+$ T cells and $CD8^+$ T cells in the breast cancer-only, in the prostate cancer-only and in mixed malignancies cohort (breast cancer and prostate cancer). In $CD4^+$ T cells and in $CD8^+$ T cells, the caspase 3 RILA assay showed a strong linear correlation with the PI RILA after 48h incubation in mixed patients ($CD4^+$: $r=0.932$, $P<0.0001$; $CD8^+$: $r=0.934$, $P<0.0001$). Similarly, in breast cancer patients, the 48h caspase 3 RILA assay had a very strong linear correlation with the 48h PI RILA in $CD4^+$ cells and $CD8^+$ cells ($CD4^+$: $r=0.937$, $P<0.0001$; $CD8^+$: $r=0.944$, $P<0.0001$). Moreover, the 48h Caspase 3 RILA assay showed a linear relationship with that of 48h PI RILA in $CD4^+$ cells and $CD8^+$ cells of prostate cancer patients ($CD4^+$: $r=0.894$, $P<0.0001$; $CD8^+$: $r=0.871$, $P<0.0001$).

Additionally, we aim to demonstrate whether the caspase-based RILA assay can save assay time compared to the PI RILA assay in $CD4^+$ and $CD8^+$ lymphocytes. In $CD8^+$ cells, 24h caspase 3 RILA assay linearly correlated with 48h PI RILA for breast cancer patients ($r=0.732$, $P<0.0001$) and mixed patients ($r=0.643$, $P<0.0001$) but only for prostate cancer patients after removing an outlier ($r=0.832$, $P=0.003$). In $CD4^+$ cells, the 24h caspase 3 RILA assay did not linearly correlate with the 48h PI RILA for breast cancer patients ($r=0.276$, $P=0.172$), prostate cancer patients ($r=0.522$, $P=0.100$) and mixed cancer patients ($r=0.251$, $P=0.134$). This implies that the 24h caspase 3 RILA assay for $CD8^+$ lymphocyte apoptosis could be an alternative for the PI RILA in breast cancer patients and prostate cancer patients.

In summary, 48h caspase 3 RILA correlated linearly with 48h PI RILA in either $CD4^+$ or $CD8^+$ T lymphocytes for not only breast cancer patients but also prostate cancer. Thus, the caspase 3 RILA

assay of breast or prostate cancer patients may be a potential alternative for the PI RILA assay. In addition, 24h caspase 3 RILA in $CD8^+$ T cells of breast and prostate cancer patients showed a strong correlation with 48h PI RILA and has therefore the potential to be an alternative to PI RILA, that may shorten post-irradiation incubation of the RILA assay by 24h and thereby increase the throughput of the assay.