hTERT methylation: a potential cancer biomarker for metastasis detection in body fluids

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Background and aim of the study

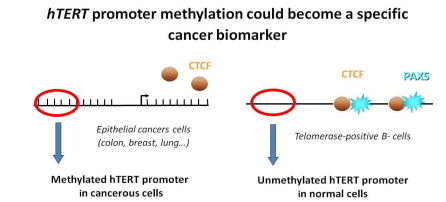
The diagnosis of **leptomeningeal metastases** (LM) is:
- essential because early treatment of LM may prevent neurological deterioration.
- based on the finding of malignant cells by cytological examination in the **cerebrospinal fluid** (CSF).
- difficult to establish by morphology alone, especially in cases with low numbers of cells.

PCR techniques are more sensitive than cytology if there is a specific genetic marker. However, most of the known markers characterize a limited set of cancers.
It would be an advantage if a single tumor marker could allow the detection of a majority of LM.

Potential marker :

- Expression of telomerase hTERT subunit might be such a marker but its use is hampered by proliferating normal lymphocyte cells, which express hTERT and thus telomerase activity (Braunschweig et al, Diagn Cytopathol 25:225–230, 2001).
- **hTERT promoter methylation** is correlated with hTERT expression in a majority of telomerase-positive tumors (*Guilleret et al, Int J Cancer 101:335, 2002*). Interestingly, hTERT promoter is unmethylated in telomerase positive normal cells, including activated lymphocytes (*Bougel et al., J Pathol 220:87-96, 2010*).

→ **Use of hTERT methylation as a biomarker might circumvent the confounding effect of activated lymphocytes.**



As the number of tumor cells can vary, a method is needed with a detection level of as low as 1% of methylated DNA in a non-methylated background.

Aim of the study:

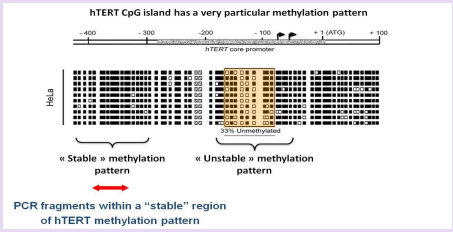
1. **To develop a specific, sensitive, quantitative, and fast method for detection of hTERT methylation**
2. **To explore its use as a cancer biomarker in the diagnosis of metastatic tumors in CSF.**

Clinical samples

- All CSF samples were immediately processed for cytological analysis. Residual CSF samples (1-2 ml) were stored at 4° C for 1-4 days before processing for hTERT methylation analysis. No patient had CSF specially and solely for this study.
- 59 CSF samples from a total of 50 patients
  - **31 CSFs** suspected for leptomeningeal metastasis (the primary tumors included 12 breast cancers, 4 medulloblastomas, 3 lung adenocarcinomas, 2 prostate carcinomas, 1 colon cancer and 1 PNET).
  - **28 CSFs** non-neoplastic samples (included inflammatory diseases and viral syndromes)
- 20 out of 23 primary tumors were hTERT methylated

Methods

Localization of PCR fragments for hTERT methylation analysis



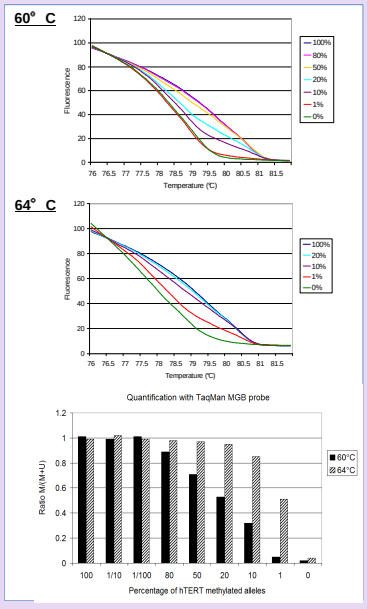
How to detect low level of hTERT methylated DNA in a non-methylated background?

Development of a real-time Methyl-Sensitive High Resolution Melting approach

Two analyses by quantitative PCR in a single tube:
1) Taqman probe specific for the methylated PCR product only (yellow channel)
2) HRM (High Resolution melt) by SYTO9 (green channel)

1-2 ml of CSF → DNA extraction (2-200 ng) → Bisulfite modification → Real-time PCR → Double analysis

Results



1. Real-time HRM development

Advantages of HRM

- scans all of the CpGs included in the target sequence
- melting curves unique for each standard sample
- simple, fast and reproducible

Increase sensitivity

1) by using PCR primers with CpGs in it (not near the 3'-end)
2) by increasing the annealing temperature of PCR (60 °C → 64°C).

2. Exploration of the Real-time HRM method in CSF samples

Positive: presence of malignant cells
Negative: malignant cell free

	Cytological diagnosis			Total
	Positive	Suspicious	Negative	
hTERT Methylated	12	4	0	16
hTERT Unmethylated	3+3*	4	4	14
Total	18	8	4	30

* 3 CSF from unmethylated hTERT tumors

- 92% (54/59 samples) of the bisulfite-modified DNAs were amplifiable by PCR and thus, analyzed by real time MS-HRM.
- None of the 24 control samples were detected positive for hTERT methylation
  → **Specificity of 100 %**
- hTERT methylation in 12 out of 15 cytologically positive samples with a hTERT methylated tumor
  → **Sensitivity of 80 %**

Conclusion

1. Real-time MS-HRM analysis = fast, sensitive, and specific technique for methylation assessment in many diagnostic and research applications.
2. **hTERT methylation only detected in the CSF from patients with a known malignancy.**
3. **hTERT methylation and cytological analyses → concordant results in 84% of the CSF samples** from patients with an hTERT methylated primary tumor (when suspicious samples were excluded).

   Sample variation could explain the discrepancy between the cytology and the methylation analysis, like aggregate formation, but also hTERT unmethylated metastasizing cells from an hTERT methylated primary tumor. The possibility of cytological overdiagnosis should also be considered.
4. Level of hTERT methylation correlated with the percentage of tumor cells estimated by cytological analysis.
5. As an adjunct to the traditional examination of CSF, the hTERT MS-HRM approach could be perform as routine diagnosis of leptomeningeal metastases.

→ hTERT methylation could become a powerful cancer biomarker in body fluids

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