Circulating nucleic acids in melanoma diagnosis

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Technical University of Munich, Freising-Weihenstephan, Germany
Definition

Circulating cell-free nucleic acids

Circulating cell-associated nucleic acids
Possible mechanism of release

- Serum/Plasma
- Lymphatic fluid
- Liquor
- Ascites
- Milk
- Urine
- Stool
- Bronchial lavage

Circulating nucleic acids
Where do they come from?

- Activated lymphocytes and other nucleated cells
- Blood-brain-barrier rupture
- Ribonucleo-protein complex
- Internucleosomal cleavage of chromatin
- Circulating nucleic acids (DNA & RNA)
- Lysis
- Necrosis
- Apoptosis
- Circulating tumor cells
- Placental/fetal cells

Two models for DNA release in the blood.

Cells undergo apoptosis and/or necrosis in cancer tissues in situ and their DNA is released into the blood stream where they undergo lysis and release their DNA content.

MALIGNANT MELANOMA

- Malignant melanoma is an aggressive, therapy-resistant malignancy of melanocytes.
- The incidence of melanoma has been steadily increasing worldwide, resulting in an increasing public health problem.
- Metastatic melanoma is characterized by no proven effective therapy and poor outcomes (mean survival ≈ 6 months)
- Malignant melanoma accounts for 5% of cutaneous tumours, but it is responsible of 80% deaths for cutaneous neoplasia

→ PREVENTION

→ EARLY DIAGNOSIS
MELANOMA PROJECT
EXPERIMENTAL PROTOCOL FOR BLOOD SAMPLES

BLOOD SAMPLE

ISET technology (RBC lysis, fixation and filtration)

RNA extraction

PLASMA

Morphology IHC

Real-time RT-PCR Tyrosinse mRNA

Total cell-free DNA BRAF V600E mut. RASSF1A methylation

Circulating Tumor cells

Circulating mRNA

Circulating DNA
Circulating plasma DNA: characteristics

- **Healthy subjects:** 0 – 100 ng/ml (average 30ng/ml corresponding to 5000 genomes per ml)
- **Cancer patients:** 0 – 1000 ng/ml (average 180ng/ml)
- Double-stranded
- In the form of nucleoprotein complex
- **Size:** 0.18 – 21 kilobases
- Rapid clearance

Total circulating DNA concentration

Circulating cell-free nucleic acids
Total circulating DNA concentration

As a tumor biomarker
Elevated level of Circulating plasma DNA is associated with cancer

Breast cancer

Circulating plasma DNA by real-time qPCR

Circulating DNA in cancer patients

- Presence of tumor specific alterations
  - point mutations,
  - microsatellite alterations,
  - SNPs,
  - hypermethylated sequences,
  - rearranged genomic sequences,
  - presence of viral DNA

- Proportion of tumor DNA: 10 – 90%
Total circulating DNA concentration → ALTERATION → GENETIC

Circulating cell-free nucleic acids
**BRAF<sup>V600E</sup> SOMATIC MUTATION IN CUTANEOUS MELANOMA**

- **exon 15** V600E (t1799a)
- glutamate → valine
- new folding: continuously activated form

- identification in ~50-70% cutaneous melanomas
- found also in NEVI

**BRAF wild-type sequence**

point mutation BRAF V600E (t1799a)
Total circulating DNA concentration → ALTERATION

GENETIC

EPIGENETIC

Circulating cell-free nucleic acids
RASSF1A HYPERMETHYLATION IN CUTANEOUS MELANOMA

RASSF1  RAS ASSOCIATION DOMAIN FAMILY 1  3p21.3

RASSF1 gene: 3 mRNA transcripts

RAS binding proteins mediating pro-apoptotic signals

RASSF1A silenced by promoter hypermethylation
Circulating cell-associated nucleic acids
**Indirect molecular method to evidence the presence of CTC in melanoma**

**TYROSINASE:**
Key enzyme for melanin synthesis

**SPECIFICITY:**
- MELANOCYTES OF HEALTHY SUBJECTS
- MELANOMA CELLS

**MARKER OF THE PRESENCE OF CIRCULATING TUMOR CELLS DERIVING FROM MELANOMA TUMORS**

1. Blood sample
2. RNA extraction
3. Real-time RT-PCR
   Tyrosinase mRNA
Blood sampling & Assay Procedure

**Real time RT-PCR**

- Blood sampling & Assay Procedure
- RNA stability in blood samples at 18-25°C

*From PAXgene Blood RNA Handbook 2005*
Tyrosinase mRNA assay sensitivity

SKMEL-28 melanoma cells
1000 – 1 cell/ml blood

y = -3.2955x + 43.1
R² = 0.8094

1 cell/ml blood

Ct

log n cell/ml blood
CLINICAL SENSITIVITY & SPECIFICITY

RESULTS

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<tr>
<th>Gene</th>
<th>SPECIFICITY</th>
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\[ \chi^2, \ p < 0.001 \]

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Qualitative analysis
\( \chi^2, \ p < 0.001 \)

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<tr>
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<td>8</td>
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Plasma total-free circulating DNA Quantitative measurement

APP

Quantitative Analysis

melanomas

Healthy controls
Measurement of total DNA concentration in plasma

APP

ng/ml blood

CONTROLS  NEVI  NON MELANOCYTIC LESIONS  MELANOMAS

\[ \neq 0 \]

\[ P < 0.05 \]

\[ P < 0.001 \]
MEASUREMENT of \(BRAF^{V600E}\) mutation in plasma

![Graph showing BRAF levels in different groups: controls, nevi, non-melanocytic lesions, and melanomas. The graph indicates a significant difference with \(P < 0.05\).]
Measurement of RASSF1A methylated form in plasma

**RASSF1A**

![Graph showing the measurement of RASSF1A methylated form in plasma. The x-axis represents different groups: Controls, Nevi, Non-Melanocytic Lesions, and Melanomas. The y-axis represents genomic copies per ml plasma. The graph indicates a significant difference (P < 0.001) between the groups.]
TYROSINASE mRNA expression in blood
CORRELATIONS

$\chi^2$ test, $p < 0.001$

<table>
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<tr>
<th>BRAF (ng/ml plasma)</th>
<th>APP</th>
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<tbody>
<tr>
<td></td>
<td>NEG</td>
<td>39</td>
<td>19</td>
</tr>
<tr>
<td></td>
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<td>19</td>
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<tr>
<td>Totale</td>
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<td>48</td>
<td>38</td>
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R = 0.58
$y = 0.978x - 1.440$

p < 0.001
$\chi^2$ test, $p < 0.05$
<table>
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<tr>
<th></th>
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<th>POSITIVE TO 2 PARAMETERS</th>
<th>POSITIVE TO 3 PARAMETERS</th>
<th>POSITIVE TO 4 PARAMETERS</th>
<th>NEGATIVE TO ALL PARAMETERS</th>
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<tr>
<td></td>
<td>(35 %)</td>
<td>(35 %)</td>
<td>(25 %)</td>
<td>(2 %)</td>
<td>(4 %)</td>
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<tr>
<td>APP</td>
<td>42%</td>
<td>74%</td>
<td>93%</td>
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<td>RASSF1A</td>
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<td>43%</td>
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**MULTIPARAMETRIC ANALYSIS**
Multiparametric panel performed on plasma compartment

![Diagram showing the relationship between APP, APP + BRAF, and APP + BRAF + RASSF1A with percentages indicating the success of the panel at each stage.](image)
Concluding remarks

Circulating Tumour Cells ↔ Circulating Nucleic acids

TUMOUR DIAGNOSIS PROGRESSION & METASTASIS