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Novel epigenetic markers of early epithelial tumor growth and prognosis

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The present work is aimed at clarifying genetic and epigenetic alterations that occur during carcinogenesis and designing perspective sets of newly identified biomarkers. The tumors of kidney, cervix, colon, ovary, and lung were analyzed in our work, using the chromosome 3 specific NotI microarrays (NMA). We have found loci/genes with essential changes in gene methylation of tumor samples. Changes in expression for these genes were confirmed. The Not-I microarray results have been used to develop epigenetic marker panels for the early detection of different tumor types (ovary and lung cancer), to discriminate the stages of tumor growth and to determine whether the tumor is metastasizing. Marker panel designing is of great perspective in clinical medicine.

Keywords: epithelial tumor, epigenetic markers, NotI-microarrays, early cancer detection, prognosis of cancer, tumor-suppressor gene.

Introduction. An important problem in oncology is the creation of sets of markers for early differential diagnosis of tumors, determining cancer progress and response to therapy. Markers, which are already in use, are effective at the later stages of tumor development. Therefore, it is necessary to intensify the search for new markers, both, genetic and epigenetic, which reflect the changes at the early stages of carcinogenesis.

The present work is aimed at clarifying genetic and epigenetic alterations that occur during carcinogenesis and designing perspective sets of newly identified biomarkers.

Microarray analysis of large-scale searches for epigenetic changes in cancers (comparison of tumor versus normal tissues). *NotI-microarray technology*. The search for tumor markers is a complicated, multistep process. The first step, usually, is the identification of large-scale genetic and/or epigenetic changes of the genome. The new type of microarrays that was recently developed in our group opens new possibilities for extensive studies of methylation patterns in normal and cancer tissues [1]. The main objective was to prepare *NotI* microarrays (NMA, *i. e*. glass microarrays with attached *NotI* DNA fragments) for comparing the normal and malignant cell genomes. Since the *NotI* enzyme cuts only unmethylated CpG pairs in the recognition site, (5'-GCGGCCGC-3'), only a small fraction of the *NotI* digested fragments (0.1–0.5 %) becomes labeled [2]. Thus, in contrast to all other methods, only digested DNA fragments were labeled. As a consequence, the probes contained 10-fold less repeats, were less sensitive to incomplete digestion, and gave less background. A simplified scheme of a *NotI* microarray analysis protocol is presented in Fig. 1.

Results and confirmation of the NotI microarray analysis of epithelial tumors. It is known, that the short arm of human chromosome 3 is involved in the development of many epithelial cancers [3–7]. Epithelial tumors make up ~ 90 % of all malignant tumors. The tumors of kidney, cervix, colon, ovary, and lung were analyzed in our work, using the chromosome 3 specific *NotI* microarrays (NMA), which contained 180 *NotI* linking clones, associated with 188 genes [8]. For all studied cancers we found genes, specifically methyla-

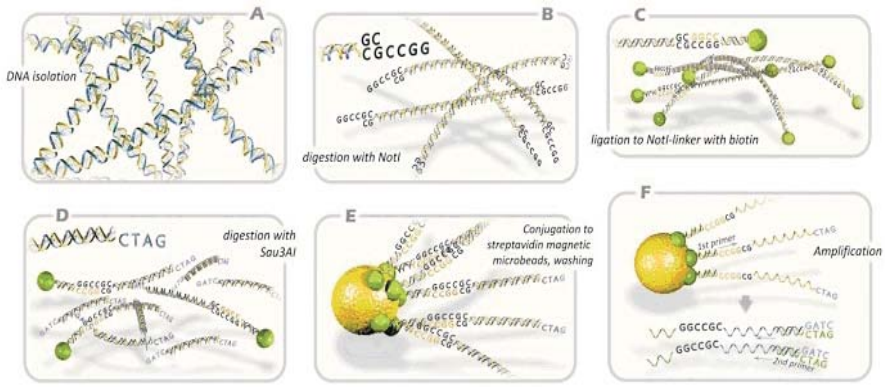

Fig. 1. A simplified scheme of the *NotI* microarray analysis protocol: *A* – isolation of genomic DNA; *B* – digestion with the methyl-specific rare-cutter enzyme *NotI*; *C* – ligation of fragments with the *NotI*-linker containing biotin; *D* – digestion with the 4-base pair recognizing restriction enzyme *Sau3AI*; *E* – conjugation to microbeads, containing streptavidin and washing; *F* – amplification of DNA sequences that have been attached to microbeads. The standard procedures were performed then: microarray hybridization, cloning, and sequencing analysis

ted in malignant cells. Many genes were methylated in a very high percentage of cancer samples. These genes can be divided into two classes: cancer specific and common for several types of cancer. The genes, *MINT24*, *BHLB2*, *GATA-2*, *RARB1*, *RBSP3*, *VHL*, *LRRC3B* [1], were involved in several cancers.

Interestingly, many methylated genes were prevoiusly unknown to be involved in the development of epithelial cancers. To prove the methylation status of genes that were observed by NMA hybridization, the methyl specific PCR (MSP) and bisulfite sequencing were performed. Genes that changed methylation status have been further investigated by relative genes expression, using Q-PCR [9].

Below we present some examples of changes in epigenetic profiles of certain genes in epithelial tumors. Primary tumors of different types of non-small cell lung carcinoma (NSCLC) were studied [10], namely, squamous cell carcinoma (SCC), and adenocarcinoma (ADC) (Fig. 2, *A*, Supplement) [11].

Results of the NMA hybridization have shown that 44 loci/genes were methylated and/or deleted with a frequency from 15 to 58 % (Fig. 2, *B*, Supplement) [11].

Methylation was observed at stage I of SCC tumors. In ADC any of the genes showed methylation patterns, characteristic for tumor progression stage.

From the frequently methylated genes by NMA, two genes were chosen and their methylation was additionally tested, using bisulfite sequencing – *VHL* (in one SCC tumor sample and one ADC –tumor sample) and *ITGA9* (in four SCC tumor samples). In all tested cases the methylation pattern was confirmed [11].

Ten genes that showed high methylation and/or deletion frequencies in NSCLC were tested for expression; these genes were expressed at the decreased level in 30–90 % of ADC cases and 38–100 % of SCC cases (Fig. 3). In general, the mRNA level in SCC samples was more frequently down-regulated than in ADC (statistically valid for *ITGA9* ($P = 0.02$) and *FOXP1* ($P = 0.05$) genes). In SCC, down-regulation was observed in 70 % of samples already at stage I, and in ADC – only in 45 %. Seven genes (except *LRRN1*, *FGD5* and *ALDH1L1*) showed increased frequencies and/or down regulated expression upon the metastasizing of ADC ($P < 0.05$ for *RBSP3* (*CTDSPL*) and *ITGA9* genes). The same tendencies were observed for other 5 genes in SCC (*IQSEC1*, *FOXP1* ($P < 0.05$), *LRRN1*, *FGD5* and *BCL6* [11]).

The NMA was used to analyze other types of cancers, namely, tumors of the cervix.

17 *NotI*-linking clones were detected that showed changes in more than 35 % of all of investigated tumor samples. The 7 of them were associated with other human cancers: *MINT24*, *BHLHB2*, *ITGA9*, *RPL15*, *RARbeta1*, *RBSP3*, and *VHL*. Some of them are accepted tumor suppressor genes, for example, *LRRC3B* and *WNT7A* [12, 13].

The 27 genes/loci showed differential expression in colorectal cancers compared with normal tissues in more than 30% of the tumor samples. [14]. The 6 genes/loci of the 27 were specific for colorectal cancer. The role of this six genes/loci (*CKLFSF6*, *PLCL2*, *LMCD1*, *NUDT16P*, *LOC131961* and *LOC650370*) in the initiation and progression of tumors remains unknown and further study is required. Several genes/loci that were identified in our study as altered genes include previously described bona fide tumor suppressor genes: *VHL*, *RBSP3*, *WNT7A*, *ITGA9*, and *FOXP1* [15].

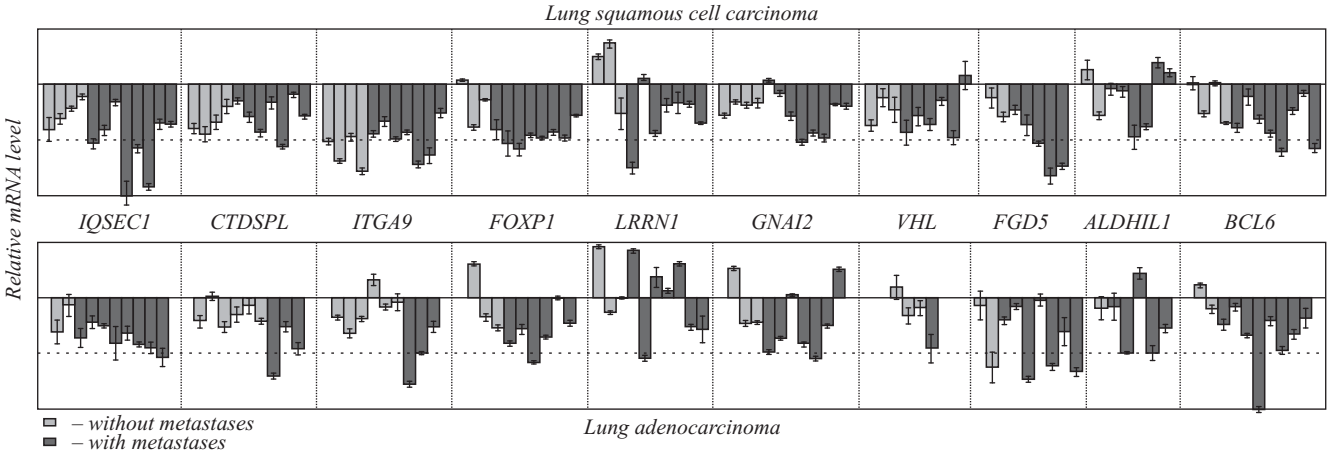

Fig. 3. Relative expression levels of 10 genes in NSCLC (SCC and ADC)

NMA of ovarian cancers has revealed that 35 genes showed the highest percentage of alterations (methylation and/or deletion). The 17 genes/loci showed changes in more than 30 % of ovarian tumor samples, regardless whether the tumors were malignant or benign [16]. The most essential changes in tumor samples were found for *LRRC3B* (80 %), *RARB* (73 %), *GATA2*, and *NKIRAS1* (66 %) genes [17]. NMA results have shown that the majority of genes with high frequency of changes in epithelial cancers are located on 3p21 and 3p24 regions [18–21].

Several genes were selected and further analyzed. For example, methylation of the *LRRC3B* gene in colorectal cancer was investigated, and, based on the obtained results, this gene was proposed as a methylsensitive gene for diagnostics [22]. Also, the expression of *LRRC3B* gene in breast, cervical, lung, RCC, ovarian, and colon cancers was tested, using Q-PCR. *LRRC3B* gene expression was unchanged in ccRCC at stages I and II, however, it was 26-fold decreased in tumors at stage III. Noteworthy, this gene did not show difference in the expression pattern in ovarian cancers without metastases, while it was 46-fold decreased in a sample with lymph node metastases (*T3N1M0*) and 107-fold less in a sample with distant metastases (*T4N1M1*) [23].

Using NMA technology the set of frequently affected genes/loci of chromosome 3 was revealed in renal cell carcinomas (RCC's) [20]. Briefly, we have identified 15 genes, which had genetic (deletion, amplification) and/or epigenetic (DNA methylation/demethylation) alterations in more than 30 % of RCC samples. Among frequently affected genes were *NKIRAS1*/*RPL15*, *MINT24*, *LRRC3B*, *VHL*, *RBSP3*, *GORASP1*, *RARB*, *NBEAL2*, *GNAI*, PPM1M, *FOXP1* and *ZIC4*. Additionally, using NMA we have detected the genetic/epigenetic changes for some cancer associated genes like *WNT7A*, *FOXP1* and *ITGA9* in ccRCC's [20].

We have performed the NMA data verification by routine methods for some of the identified genes. Thus, we showed hypermethylation of the *LRRC3B* promoter CpG-island in 43 % of clear cell RCC's [12]. Moreover, the restoration of *LRRC3B* expression led to a significant decrease of colony formation by KRC/Y cell line [23].

It was identified that the *NKIRAS1* expression was down-regulated in 75 % of ccRCC samples (9 of 12) compared to surrounding normal tissue. This was accompanied by copy number change of the NKIRAS1 gene, which was observed in 64 % (9 of 14) of ccRCC samples [17]. Hypermethylation and high deletion frequency of the *WNT7A* gene were detected in 66 % (29/44) and 85% (23/27) of analyzed ccRCC's. In addition, the re-expression of *WNT7A* led to inhibition of the proliferation rate in RCC cell lines [13]. Therefore, application of NMA technology contributed to identification of the frequently hypermethylated genes in ccRCC.

Subsequently we have used the newly detected *LRRC3B* gene in combination with well-known hypermethylated genes such as *APC* and *FHIT* to create a methylation-based biomarker panel for diagnostics of RCC [24–26]. We have detected hypermethylation of *LRRC3B*, *FHIT* and *APC* genes in 33.3 %, 27.8 % and 33.8 % of plasma samples of the RCC patients. These data emphasize the perspective application of NMA te-

Table 1
Detection and discrimination of NSCLC groups with different histological characteristics, using the set of 19 selected genes

Group	Use	Sets of markers
A	Detection of NSCLC	*IQSEC1*, *GORASP1*/*TTC21A*, *NKIRAS1*/*RPL15*, *RBSP3* (*CTDSPL*), *LRRN1*, *ITGA9*, *FOXP1*, *TRH*; Sp* = (95 ± 3) %; Sn** = (85 ± 6) %; P*** < 0.01
B	Discrimination ADC and SCC	*NKIRAS1*/*RPL15*, *CGGBP1*, *VHL*, *LOC28375*, *EPHB1*, *BHLHE40*, *ANKRD28*; Sp = (83 ± 11) %; Sn = (79 ± 8) %; P = 0.01
C	Detection of metastases in ADC	*LRRC3B*, *UBE2E2*, *WNT7A*, *FGD5*; Sp = 100 %; Sn = (80 ± 18) %; P = 0.02
D	Detection of metastases in SCC	*GORAST1*/*TTC21A*, *LRRN1*, *VHL*, *MITF*; Sp = (86 ± 13) %; Sn = (86 ± 8) %; P < 0.01

N o t e. Sp* – specificity; Sn** – sensitivity of the set; P*** – parameter shows significance of compared groups distinction, calculated using Fisher exact test and χ^2 criteria.

chnology for identification of new methylation-based biomarkers [27].

Selection of genes/biomarkers for detection and status of epithelial cancers. Thus, we have completed the first stage of the search for biomarkers. We found a number of epigenetic changes in certain genes in different tumor types. The next step was to select genes for biomarker panels. One of the examples of such panels to distinguish benign and malignant tumors was the *NotI*-linking clones forming DNA-microarray chips. These panels were used to investigate lung and ovary cancers [11, 16].

If methylation and/or deletion were found in two or more genes of a biomarker panel, such sets would be then recognized as specific groups. In this way, the NMA results afforded an opportunity to select some of the genes with high levels of epigenetic changes for the next stage of marker detection. A cluster analysis permits the putative gene selection to discriminate different stages of cancerogenesis. Based on our results, we can propose a set of 8 genes for detection of *NSCLC* in lung biopsies of all stages, including stage I (Table 1, A) [11]. To select the genes that can help to discriminate ADC without metastases from the three other groups, i. e., ADC with metastases, SCC without and with metastases, was quite easy, using [the] data, obtained by *NotI* microarray analysis. To distinguish ADC from SCC, the most promising set should include *NKIRAS1*/ *RPL15*, *CGGBP1*, *VHL*, *LOC285375*, *EPHB1*, *BHL HE40*, and *ANKRD28* biomarkers (Table 1, B) [11].

For discrimination between ADC without and with metastases, it is possible to use *LRRC3B*, *UBE2E2*, *WNT7A*, and *FGD5* set of biomarkers (Table 1, C) [11].

Using the obtained *NotI* microarrays data with comparatively small number of samples, it was quite difficult to discriminate between SCC cases without and with metastases (Table 1, D) [11]. The most suitable set consists of only three genes (*GORASP1*/*TTC21A*, *LRRN1*, and *VHL*) that showed increased frequencies of methylation and/or deletion upon progression of SCC, and one gene – *MITF* showed an opposite trend, i. e. the decreased frequency of methylation and/or deletion. If deletions and/or methylation were not detected for any of the three down-regulated genes, one point was given for each gene in which methylation was not found (no point counts if changes were detected) in the sample [11].

Concluding, a comprehensive statistical analysis suggested a set of 19 gene markers for the early detection, characteristics of tumor progression, prognosis of metastasizing, and for discrimination between SCC and ADC with sensitivity and specificity of 80–100 % (Table 1, A–D) [11].

For detection of cancer cells in ovarian biopsies of all stages, including early one, the most perspective set from analyzed genes was the set of 8 biomarkers (Table 2, A) [16].

For discrimination between benign and malignant tumors it is possible to use the set of 8 biomarkers (Table 2, B) [16].

To distinguish between stages I + II and stages III + + IV of ovarian cancer the most promising set should include 5 biomarkers (Table 2, C) [16].

Summarizing the obtained data, the set of 10 gene markers was developed for early detection and discrimination of ovarian cancer groups with different histological characteristics [16].

Table 2
Early detection and discrimination of ovarian cancer groups with different histological characteristics, using the set of 11 selected gene

Group	Use	Sets of markers
A	Early detection of ovarian cancer	*NKIRAS1/RPL15, THRB, RBSP3 (CTDSPL), IQSEC1, NBEAL2, ZIC4, LOC285205, PAQR9*; Sp* = (94 ± 5) %; Sn** = (72 ± 11) %; P*** < 0.01
B	Discrimination of benign tumors and cancer	*NKIRAS1/RPL15, THRB, RBPS3 (CTDSPL), IQSEC1, NBEAL2, ZIC4, LOC285205, PAQR9*; Sp = (71 ± 5) %; Sn = (72 ± 11) %; P = 0.04
C	Discrimination of I + II stages and III + IV stages	*LOC285205, CGGBP1, EPHB1, PAQR9, NKIRAS1/RPL15*; Sp = (88 ± 12)% Sn = (70 ± 14) %; P < 0.01

N o t e. Sp* – specificity; Sn** – sensitivity of the set; P*** – parameter shows significance of compared groups distinction, calculated using Fisher exact test and χ^2 criteria.

Conclusions. Different types of epithelial tumors were analyzed, using the *NotI* microarray technology. We have found loci/genes with essential changes in expression and methylation in a high proportion of tumor samples. Changes in the expression for several genes were confirmed, using the bisulfite sequencing (to monitor changes in DNA methylation) and Q-PCR (to assess the relative level of gene expression).

Thus, the *NotI* microarray technology allows the development of a panel of specific markers for the early detection of different tumor types as well as to discriminate the stages of tumor growth and to determine whether the tumor is metastasizing.

The case study of lung and ovary tumors was a bright example of utility of marker panels that were created for detection of lung carcinoma, differentiation between adenocarcinoma and squamous lung carcinoma, and tumors with or without metastases. In the case of ovary carcinoma, the marker panels were created for early detection, discrimination of benign and malignant tumors, and also for discrimination of stages I + II *vs* stages III + IV. In sum, we developed a new approach to search for epigenetic markers of epithelial cancers. The presented results have a great perspective for their use in clinical medicine.

В. В. Гордіюк, О. Г. Кондратов, Г. В. Геращенко, В. І. Кашуба

Нові епігенетичні маркери ранньої детекції пухлин епітеліального походження та їх прогнозування

Резюме

В огляді розглянуто генетичні та епігенетичних зміни, які відбуваються при утворенні пухлин, та пошук перспективних наборів нових біомаркерів. Представлено дані NotI-мікрочіпів для 3-ї хромосоми людини щодо змін у пухлинах нирок, шийки матки, товстого кишечника, яєчників і легень. Знайдено локуси/гени з істотними змінами метилювання у зразках пухлин, які супроводжуються зниженням експресії відповідних генів. Результати мікрочіпів використано для розробки панелей епігенетичних маркерів ранньої детекції різних типів пухлин (яєчників і легенів), а також для розрізнення ступенів прогресії пухлин і виявлення метастазів. Створення подібних панелей маркерів є перспективним для застосування в клінічній медицині.

Ключові слова: пухлини епітеліального походження, епігенетичні маркери, NotI-мікрочіпи, рання детекція раку, прогнозування раку, гени – супресори росту пухлин.

В. В. Гордиюк, А. Г. Кондратов, А. В. Геращенко, В. И. Кашуба

Новые эпигенетические маркеры ранней диагностики опухолей эпителиального происхождения и их прогнозирования

Резюме

В обзоре рассмотрены генетические и эпигенетические изменения,сопутствующие образованию опухолей, и поиск перспективных наборов новых биомаркеров. Представлены данные NotI-микрочипов для 3-й хромосомы человека относительно изменений в опухолях почек, шейки матки, толстого кишечника, яичников и легких. Найдены локусы/гены с существенными изменениями метилирования в образцах опухолей, сопровождающимися снижением экспрессии соответствующих генов. Результаты микрочипов использованы для разработки панелей эпигенетических маркеров ранней детекции разних типов опухолей (яичников и легких), а также для дифференцирования степени прогрессии опухолей и выявления метастазов. Создание подобных панелей маркеров является перспективным для применения в клинической медицине.

Ключевые слова: опухоли эпителиального происхождения, эпигенетические маркеры, NotI-микрочипи, ранняя детекция рака, прогнозирование рака, гены – супресоры роста опухолей.

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