Prognostic significance of CpG island methylator phenotype in surgically resected small cell lung carcinoma

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DNA methylation-associated gene silencing is a common event in human cancers, including primary lung cancer. 1–3 Global hypomethylation is thought to play a role in carcinogenesis of primary lung cancer by increasing chromosome instability. 1–3 However, local hypermethylation of promoter CpG islands consistently inactivates their downstream genes, including tumor-suppressor genes. The CpG island methylator phenotype (CIMP) displays characteristic alterations of promoter DNA methylation in colorectal cancer, 4 glioblastoma 5 and breast cancer. 6 Poirier et al. 7 report on genome-wide DNA methylation in SCLC, by using 34 fresh-frozen primary tumors, six distinct primary patient-derived xenografts and seven cell lines. In their study, the methylation patterns were clearly tied to each gene expression, and DNA methylation profiling successfully distinguished subtypes of primary SCLC tumors. However, they mentioned no clinical feature by subdividing SCLC tumors. Here we set out a program to establish a clinically-useful subclassification of SCLC using a CIMP status. Our ultimate goal is to clarify the clinical importance of the molecular biological classification.

Materials and Methods

Subjects and tumor samples. Between 1 July 1995 and 30 September 2009, a total of 1873 patients with primary lung cancer underwent surgical resection at the Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo, including 49 (2.6%) SCLC patients. Among these cases, we excluded combined type SCLC tumors based on diagnosis by expert pathologists (NM and YI) using World Health Organization classification. In addition, we found that the tissue amount of 21 cases was insufficient for further molecular experiments. Subsequently, we were able to use 28 samples for methylation analysis, and 25 samples were available for gene expression analysis.
Cases with atypical histology were examined by a panel of Japanese expert lung pathologists organized by a neuroendocrine tumor study group, and tumors with consensus diagnosis were used. Written informed consent for medical research was obtained from all patients. Clinical and pathological data were stored in a database in accordance with hospital privacy rules. The study protocol was approved by the institutional review board of JFCR.

**Illumina Infinium methylation assay and expression microarray analysis.** After bisulfite conversion of genomic DNA, 28 samples were analyzed using Illumina’s Infinium Human Methylation27 Beadchip Kit (WG-311-1202), which contains 27 578 CpG loci covering more than 14 830 human RefSeq genes at single-nucleotide resolution. All microarray datasets have been deposited into the NCBI GEO database (accession number GSE50412). All statistical analyses were carried out using β-values: (signal intensity of methylated probe)/(signal intensity of methylated probe + signal intensity of non-methylated probe), which were quantified using M-values that were calculated as the base 2 logarithm ratio of the intensities of the methylated and unmethylated probes. (10)

The RNA integrity number (RIN) index was calculated for each sample using Agilent 2100 Expert software, and only RNA samples with RIN number ≥ 4 were further processed. Finally, the RIN expression in 25 SCLC tissue samples was analyzed by gene expression microarray (SurePrint G3 Human Gene Expression Microarray Kit 8 × 60M; Agilent Technologies, Santa Clara, CA, USA). The datasets of mRNA expression have been deposited into the NCBI GEO database (accession number GSE66202).

**Statistical analysis and validation using in-silico datasets.** Unsupervised hierarchical clustering analysis was performed by using the Euclidean distance and complete linkage algorithm on Cluster 3.0, and the dendrogram and heat map were constructed using TreeView (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). To reconfirm the results of hierarchical clustering, we performed non-negative matrix factorization (NMF), a kind of consensus clustering, by using the GenePattern module (http://www.broadinstitute.org/cancer/software/genepattern#). Previously, using the software, we successfully obtained robust results of clustering for squamous cell carcinomas and adenocarcinomas of the lung. Here we selected the number of clusters (k = 2) on NMF, based on the cophenetic correlation coefficients (k) given by the software. NMF has several advantages: it is more stable than the self-organizing map and hierarchical clustering, as well as numbers of clusters are given by the cophenetic correlation coefficients, which means the clustering is more objective, as detailed by Fujiwara et al. (14) Categorical data were compared using the χ²-test or Fisher’s exact test. A non-parametric approach (Wilcoxon rank-sum test) was used to determine probes/genes that are differentially methylated between the two groups of interest. Survival curves were calculated using the Kaplan–Meier method and survival distributions were compared with a log-rank test, using SPSS version 22 software (SPSS, Chicago, IL, USA).

**Results**

**Clinicopathology of patients with small cell lung cancer.** Clinicopathological characteristics of 28 patients examined are summarized in Table 1. They were mostly male (20/28, 71%) and 24 (86%) underwent lobectomy or more extensive surgery. Of the 28 patients, 12 (43%) were node-negative. 11 of whom had p-stage I tumors, and overall 5-year survival was 69.4%. Of the 28 cases enrolled, both tumor and normal lung tissues were available for 13 cases. So, we used the 13 paired tissues for comparison studies between tumor and normal tissues. We focused on surgical SCLC cases in this study because such cases may include early-stage as well as advanced disease.

**Table 1. Characteristics of surgically-treated patients with small cell lung cancer examined in this study**

| Characteristic | Cluster 1 | Cluster 2 | P-value |
|---------------|-----------|-----------|---------|
| Number (n = 28) | 9 | 19 | 0.547 |
| Age, years | 68.6 ± 5.9 | 67.0 ± 6.8 | 0.039 |
| Gender, male | 4 (44%) | 16 (84%) | 0.009 |
| Smoking (pack years) | 33.8 ± 22.4 | 50.8 ± 22.2 | 0.291 |
| Chemotherapy | | | 0.337 |
| Preoperative | 4 (44%) | 5 (26%) | 0.337 |
| Postoperative | 6 (67%) | 16 (84%) | 0.291 |
| Surgical procedure | | | 0.741 |
| Limited surgery | 1 (11%) | 3 (16%) | 0.227 |
| pT factor (T1/T2/T3/T4) | 3/4/1/1 | 13/5/1/0 | 0.721 |
| pN factor (N0/N1/N2) | 3/3/3 | 9/6/4 | 0.1802 |
| IHC stain (positive) | | | 0.678 |
| (1) Each marker | | | 0.138 |
| Chromogranin A | 8 (89%) | 9 (53%) | 0.067 |
| Synaptophysin | 8 (89%) | 10 (59%) | 0.114 |
| CDS5/NCAM | 9 (100%) | 14 (82%) | 0.1802 |
| (2) All three markers | | | 0.075 |
| All positive | 7 (78%) | 7 (41%) | 0.075 |
| One or two positive | 2 (22%) | 8 (80%) | 0.216 |
| ly (positive/negative/NA) | 9/0/0 | 11/7/1 | 0.030 |
| v (positive/negative/NA) | 8/1/0 | 15/3/1 | 0.702 |
| p (positive/negative/NA) | 5/4/0 | 5/14/0 | 0.132 |
| pm (positive/negative/NA) | 2/7/0 | 0/19/0 | 0.033 |

Data of age and smoking: mean ± SD. IHC, immunohistochemical.
advanced-stage cases. Tumors with advanced stages are usually found in patients whose tumors are unresectable and only biopsy specimens are available.

**Comparisons of DNA methylation patterns between tumors and normal tissues, using both our own data and in-silico data for validation analysis.** After genome-wide DNA methylation sequencing of 28 tumor tissues and 13 normal lung tissues, we found 2397 that had an SD of mean β-value that met the >0.2 threshold within the 13 tumor sample set. To identify differences of global methylation patterns between cancerous and normal tissues, β-values of the 13 paired tumor and normal tissues were analyzed using the hierarchical clustering and the NMF. Among of 2397 sites with an SD of mean β-value larger than 0.2 within tumor samples, 147 candidate sites were selected and analyzed by excluding the sites with no important statistical differences between tumor and normal tissues (Mann–Whitney U-test, P < 0.05), and values (β-value of tumor − [β-value of normal tissue]) smaller than 0.01 in 2 of 13 tumors (Table S1). As shown in Figure 1(a,b), the results were very similar for the two clustering methods, and tumor and normal samples were clearly clustered, implying that the clusters were quite robust. Gene ontology analyses of the 2397 loci implicated three main pathways involved in the etiology of these cancers: neuroactive ligand-receptor interaction, calcium signaling pathway and gap junction (Table S2).

**The validity of DNA methylation patterns between cancerous and normal tissues in independent cohorts obtained from the GEO database.** Using the 147 genes mentioned above, we verified this gene set to be discriminable between malignant and benign lung tissues. The DNA methylation datasets of tumors and normal lung tissues reported previously\(^{(17)}\) were used as an independent validation set: GSE35341 downloaded from NCBI GEO data repository http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35341. We employed 18 SCLC tumor tissues (T1–T18) and five normal lung tissues (N1–N5) from the series to confirm our results. To apply the probes of the Illumina Infinium HumanMethylation27 microarray to that of the NimbleGen tiling arrays, all sequences of each probe set were compared and adjusted approximately. According to the result of clustering analysis in GSE35341, the 147 probes were able to distinguish cancerous from non-cancerous specimens sufficiently: 17 out of 18 SCLC were correctly classified as “tumor” and 4 out of 5 normal lung tissues were correctly classified as “healthy” (Fig. 2).

**Subclassification of small cell lung cancer by methylation patterns.** Next, to identify SCLC subgroups, we used the 1741 probes with an SD of mean β-value in tumor tissues >0.2 on each site and performed both the unsupervised hierarchical clustering (Fig. 3a) and the NMF (Fig. 3b). We identified two clusters with different methylation levels: Cluster 1 (n = 9) and Cluster 2 (n = 19). As shown in Figure 3(a,b), the two clusters created by both the methods were exactly the same, implying that the clusters were very robust. Cluster 1 tumors were identified as SCLC CIMP and Cluster 2 was non-CIMP, because the CpG islands of Cluster 1 tumors were significantly hypermethylated as compared with Cluster 2 tumors (Fig. 4).

**Clinical, pathological and immunohistochemical data of SCLC in the two subgroups are summarized in Table 1.** Comparison of the baseline characteristics between these two groups showed significant differences in gender, ly and pm factors. Furthermore, tumors of Cluster 2 tended to have a less neuroendocrine nature, such as chromogranin A (89% Cluster 1 vs 53% Cluster 2), synaptophysin (89% vs 59%) and all markers (78% vs 41%), although the tendencies were not statistically significant, which agrees with our previous study results based on neuroendocrine marker expression.\(^{(19)}\)

**Survival analysis.** During the median follow-up period of 37.4 months, 16 patients suffered cancer relapse: 8 (8/9, 89%) in Cluster 1 and 8 (8/19, 42%) in Cluster 2. The 5-year disease-free survival (DFS) rate for the entire group was 46.2%, and the 5-year DFS of Cluster 1 (11.1%) was much lower than that of Cluster 2 (62.7%). These differences are highly significant (Fig. 5, P = 0.002).

**Univariate and multivariate analyses for prognosis.** Next we performed multivariate analyses for prognosis, by using variables with a value of P < 0.10 in the univariate analyses (Table 2). Postoperative chemotherapy was a significant good prognostic factor, and being a Cluster 1 patient (SCLC CIMP) was a poor prognostic factor (Table 3).

**Correlation of gene expression with their methylation patterns, and pathway analysis.** Differentially expressed genes were identified by comparing Cluster 1 (n = 9) and Cluster 2 (n = 16). To validate if methylated genes were actually down-regulated, we performed multivariate analyses for prognosis, by using variables with a value of P < 0.10 in the univariate analyses (Table 2). Postoperative chemotherapy was a significant good prognostic factor, and being a Cluster 1 patient (SCLC CIMP) was a poor prognostic factor (Table 3).

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![Fig. 1. Hierarchical clustering and non-negative matrix factorization of paired 13 small cell lung cancer (SCLC) tumors and 13 normal tissue samples. Unsupervised clustering of 26 samples was performed using 147 variably methylated loci. (a) Hierarchical clustering displays relative methylation levels (red, more methylated; green, less methylated). Except for three tumors, clustering analysis classified tumors and normal tissues. (b) Non-negative matrix factorization (K = 2) distinguished tumors and normal tissue except for two tumors. Samples (n = 26) are listed in the same order along the x and y axes.](image-url)
regulated or not, we examined correlation of methylation with expression of genes by using the 1741 methylation data with the gene expression datasets of 58 724 probes. 1530 CpG sites (corresponding to 1220 genes) were identified, and 46 genes of them (corresponding to 55 CpG loci) were negatively correlated with the FDR-adjusted \( P \)-value \(< 0.05\) (Table S3). Furthermore, tumor necrosis factors (TNFRSF10A and TNFSF8) and apoptosis factors (TANK and TRADD) were closely related to the poor prognosis of Cluster 1 patients.

The functional enrichment analysis for these genes revealed three biological pathways. Two of them were expressed with significant differences and are listed in Table S4, termed three biological pathways. This implies that apoptotic activities and characteristics related to non-small cell carcinoma (NSCLC) are reduced in Cluster 1 tumors by hypermethylation.

**Discussion**

High throughput methylation platforms enable us to reveal extensive methylation profiling of a large number of genes for a variety of human cancers, including SCLC. In this study, we found that the 147 probes could be a molecular classifier between cancerous and non-cancerous specimens (Fig. 1). In addition, our functional annotation analysis demonstrated close relevance between SCLC tumors and the neuroactive ligand-receptor interaction pathway. Considering both the reports of Kalari et al. and the present report, loss of proper neuronal differentiation may be involved in the progression process of carcinoma cells to a more highly malignant stage (i.e., SCLC).

Previously, we identified two subgroups with different clinical outcomes by gene expression profiling using 38 surgically resected high-grade neuroendocrine tumors, including 15 pure type SCLC tumors\(^7\), and confirmed by immunohistochemistry of neuroendocrine markers\(^{20}\). Of the two subgroups, one subset (termed non-HGNT2) showed significantly worse prognosis compared with another (5-year survival 12\% vs 83\%; \( P = 0.0094\)). Here, by applying DNA methylation profiling techniques, we obtained two clusters with different methylation patterns and different prognosis: Cluster 1 with global high CIMP and poor prognosis (SCLC CIMP) and Cluster 2 with low CIMP and better prognosis (non-CIMP). Poirier et al.\(^7\) also observed distinct subtypes of SCLC by using a DNA methylation profiling technique. In their study, the three clusters (termed M1, M2 and SQ-P) were identified with each different methylation pattern as well as with distinct gene expression. They confirmed that two subtypes (M1 and M2) were significantly more frequently methylated compared with the SQ-P. They discussed the relationship between the methylation status and the biological aggressiveness of tumor itself, but did not show any clinical data linked with the results. We successfully showed that the increasing methylation level was related to the poor prognosis. This suggests the possibility that
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A number of genes with higher methylation levels and downregulated gene expression proved to be involved in the apoptosis and NSCLC pathways. To our knowledge, there are few reports describing that these pathways might be strongly related with the therapeutic results of SCLC. The apoptotic pathway plays an essential role in the development and maintenance of tissue homeostasis, and has an important role in carcinogenesis, cancer progression and resistance to anti-cancer agents. It has been reported that the apoptosis pathway can be effectively inactivated in SCLC cells. The authors of these reports found resistance to FasL and TRAIL-induced death of SCLC cells, and identified silencing of Fas, TRAIL-R1 and caspase-8 expression caused by DNA methylation. In the present study, we found that SCLC CIMP had a particularly worse outcome compared with that of non-CIMP, and showed markedly lower expression of TNFRSF1A, TNFRSF10A, and TRADD. Among them, TNFRSF10A was the most frequently hypermethylated and downregulated gene, inactivation of which was also reported previously in osteosarcomas, gastric carcinomas and glioblastoma multiforme. These results strongly suggested an important prognostic role of epigenetic suppression of TRADD.

Table 2. Results of univariate analysis of prognostic factors influencing disease-free survival

| Variable                  | Hazard ratio | 95% CI       | P-value |
|---------------------------|--------------|--------------|---------|
| Age, years                | 0.972        | (0.888, 1.064)| 0.539   |
| Gender, male              | 0.641        | (0.221, 1.862)| 0.414   |
| Smoking                   | 0.979        | (0.956, 1.002)| 0.079   |
| Preoperative chemotherapy  | 2.593        | (0.926, 7.264)| 0.070   |
| Postoperative chemotherapy| 0.198        | (0.069, 0.565)| 0.002   |
| Limited surgery           | 1.377        | (0.387, 4.897)| 0.621   |
| pT factor                 | 1.853        | (1.025, 3.350)| 0.041   |
| pN factor                 | 1.518        | (0.819, 2.814)| 0.185   |
| NE marker immunostaining  |              |              |         |
| Chromogranin A            | 3.011        | (0.853, 10.62)| 0.087   |
| Synaptophysin             | 2.514        | (0.712, 8.875)| 0.152   |
| NCAM†                     | 27.68        | (0.099, 7707)| 0.248   |
| All positive              | 2.111        | (0.732, 6.095)| 0.167   |
| pl 1-3                    | 1.347        | (0.488, 3.723)| 0.565   |
| pm (+)                    | 4.858        | (0.970, 24.34)| 0.055   |
| v (+)                     | 3.062        | (0.402, 23.31)| 0.280   |
| ly (+)                    | 2.041        | (0.580, 7.186)| 0.267   |
| Cluster 1 (SCLC CIMP)     | 4.399        | (1.578, 12.27)| 0.005   |

Table 3. Results of multivariate analysis of prognostic factors influencing disease-free survival

| Variable                  | Hazard ratio | 95% CI       | P-value |
|---------------------------|--------------|--------------|---------|
| Postoperative chemotherapy| 0.179        | (0.057, 0.557)| 0.003   |
| Cluster 1 (SCLC CIMP)     | 4.708        | (1.553, 14.27)| 0.006   |

It could be useful for surgical indication and for determining adjuvant therapy.

A number of genes with higher methylation levels and downregulated gene expression proved to be involved in the apoptosis and NSCLC pathways. To our knowledge, there are few reports describing that these pathways might be strongly related with the therapeutic results of SCLC. The apoptotic pathway plays an essential role in the development and maintenance of tissue homeostasis, and has an important role in carcinogenesis, cancer progression and resistance to anti-cancer agents. It has been reported that the apoptosis pathway can be effectively inactivated in SCLC cells. The authors of these reports found resistance to FasL and TRAIL-induced death of SCLC cells, and identified silencing of Fas, TRAIL-R1 and caspase-8 expression caused by DNA methylation. In the present study, we found that SCLC CIMP had a particularly worse outcome compared with that of non-CIMP, and showed markedly lower expression of TNFRSF1A, TNFRSF10A and TRADD. Among them, TNFRSF10A was the most frequently hypermethylated and downregulated gene, inactivation of which was also reported previously in osteosarcomas, gastric carcinomas and glioblastoma multiforme. These results strongly suggested an important prognostic role of epigenetic suppression of TRADD.

This is the first paper that describes the 5-year DFS rates of distinct molecular SCLC subgroups (SCLC CIMP and non-CIMP). However, there were some limitations of our study: (i) the small number of cases; (ii) all samples were surgically resected; (iii) there was no validation set; and (iv) the influence of preoperative chemotherapy. Because the mainstream treatment for SCLC patients is chemo-radiotherapy or chemotherapy alone, surgical treatment is seldom undertaken. Therefore, we rarely obtain sufficient fresh materials for research. Even when samples can be obtained, preoperative chemotherapy makes analyses difficult. In this study, 9 (32%) of 28 SCLC patients underwent chemotherapy before surgery. To maintain
the statistical power, we could not avoid excluding these cases. Similarly, we could not design an additional validation test. In future we need further studies with increased numbers of cases, including extended and pretreated tumors.

In summary, we revealed that SCLC CIMP could be an important prognostic indicator after surgical treatment. This may be a useful resource for surgical indication for SCLC patients, especially with CT1NOMO stage tumors, and may help in the development of novel chemotherapeutic agents, including demethylating agents.

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Disclosure Statement

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References

1. Esteller M. Epigenetics in cancer. N Engl J Med 2008; 358: 1148–59.
2. Issa J-P. Aging, DNA methylation and cancer. Crit Rev Oncol Hematol 1999; 32: 31–43.
3. Herman JG. Epigenetics in lung cancer: focus on progression and early lesions. Chest 2004; 125: 119S–22S.
4. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 1999; 96: 5681–6.
5. Noshmehr H, Weisenberger DJ, Diefes K et al. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. Cancer Cell 2010; 17: 510–22.
6. Fang F, Turcan S, Rimner A et al. Breast cancer methylomes establish an epigenomic foundation for metastasis. Sci Transl Med 2011; 3: 75ra25.
7. Poirier JT, Gardner EE, Conns N et al. DNA methylation in small cell lung cancer defines distinct disease subtypes and correlates with high expression of EZH2. Oncogene 2015; 9: 1–10.
8. Travis WD, Nicholson S, Hirsch FR et al. Small cell carcinoma. In: Travis WD, Brambilla E, Mueller-Hermelink HK, Harris CC, eds. Pathology and Genetics, Lyon: IARC, 2004; 31–44.
9. Asamura H, Kameya T, Matsumo Y et al. Neuroendocrine neoplasms of the lung: a prognostic spectrum. J Clin Oncol 2006; 24: 70–6.
10. Du P, Zhang X, Huang CC et al. Comparison of β-value and M-value methods for quantifying methylation levels by microarray analysis. BMC Bioinformatics 2010; 11: 587.
11. Lee DD, Seung HS. Learning the parts of objects by non-negative matrix factorization. Nature 1999; 401: 788–91.
12. Brunet JP, Tamayo P, Golub TR, Mesirov JP. Metagenes and molecular pattern discovery using matrix factorization. Proc Natl Acad Sci USA 2004; 101: 4164–9.
13. Inamura K, Fujiwara T, Hoshida Y et al. Two subclasses of lung squamous cell carcinoma with different gene expression profiles and prognosis identified by hierarchical clustering and non-negative matrix factorization. Oncogene 2005; 24: 7105–13.
14. Fujiwara T, Hiramatsu M, Isagawa T et al. ASCL1-coexpression profiling but not single gene expression profiling defines lung adenosquamous of neuroendocrine nature with poor prognosis. Lung Cancer 2012; 75: 119–25.
15. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003; 19: 185–93.
16. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Statist Soc Ser B (Methodol) 1995; 57: 289–300.
17. Kalaris S, Jung M, Kernstine KH, Takahashi T, Pfeifer GP. The DNA methylation landscape of small cell lung cancer suggests a differentiation defect of neuroendocrine cells. Oncogene 2013; 32: 3559–68.
18. da Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44–57.
19. Jones MH, Vrtanen C, Honjo D et al. Two prognostically significant subtypes of high-grade lung neuroendocrine tumours independent of small-cell and large-cell neuroendocrine carcinomas identified by gene expression profiles. Lancet 2004; 363: 775–81.
20. Hamaanaka W, Motoi N, Ishikawa S et al. A subset of small cell lung cancer with low neuroendocrine expression and good prognosis: a comparison study of surgical cases to inoperable cases with biopsy. Hum Pathol 2014; 45: 1045–56.
21. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer 2009; 9: 501–7.
22. Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp Cell Res 2000; 256: 42–9.
23. Hopkins-Donaldson S, Ziegler A, Kurtz S et al. Silencing of death receptor and caspase-8 expression in small cell lung carcinoma cell lines and tumors by DNA methylation. Cell Death Differ 2003; 10: 356–64.
24. Kaminsky VO, Surova OV, Vaculova A, Zhivotovsky B. Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. Carcinogenesis 2011; 32: 1450–8.
25. Sadikovic B, Yoshimoto M, Chilton-MacNeill S, Thorner P, Squire JA, Zie- lenska M. Identification of interactive networks of gene expression associated with osteosarcoma oncogenesis by integrated molecular profiling. Hum Mol Genet 2009; 18: 1962–75.
26. Lee KH, Lim SW, Kim HG et al. Lack of death receptor 4 (DR4) expression through gene promoter methylation in gastric carcinoma. Langenbecks Arch Surg 2009; 394: 661–70.
27. Martinez R, Setien F, Voelter C et al. CpG island promoter hypermethylation of the pro-apoptotic gene caspase-8 is a common hallmark of relapsed glioblastoma multiforme. Carcinogenesis 2007; 28: 1264–8.