Genomic copy number alterations associated with the early brain metastasis of non-small cell lung cancer

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Abstract. Frequent early development of systemic metastasis leads to unfavourable clinical prognosis of non-small cell lung cancer (NSCLC). Although brain metastasis (BM) contributes significantly to morbidity and mortality of NSCLC, relevant driver mechanisms are largely unknown. To elucidate genetic alterations associated with early BM of NSCLC, we retrospectively collected 18 NSCLC cases with BM [12 adenocarcinomas (ADC) and 6 squamous cell carcinomas (SQCC)] whose surgical tissues of both primary and brain metastatic tumors were preserved as formaldehyde-fixed and paraffin-embedded (FFPE) pathological samples. When chromosomal copy number alterations (CNA) of those FFPE samples were analysed by the Molecular Inversion Probe (MIP) technology, the most frequent CNAs detected in primary lung ADCs were gains of 3q, 5p, 5q, 6p, 8q, 9p, 11p, 15q, 17q and losses of 10q and 22q whereas primary lung SQCCs revealed gains in 4q and 12q and loss in 9q. In particular, when comparative MIP was performed in primary 12 ADCs depending on the pattern of BM to uncover predetermining signatures that can predict the risk of BM, selectively amplified regions of primary lung ADCs (5q35, 10q23 and 17q23-24) were identified as significantly associated with the development of early BM within 3 months after first diagnosis of primary tumors. Those regions harbour several candidate genes including NeurL1B, ACTA2, FAS and ICAM2. Although more validation is needed, the genetic signatures elucidated in this study help to identify useful molecular markers defining an NSCLC patient subgroup at risk of early BM, guiding therapeutic decisions.

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Introduction

Non-small cell lung cancer (NSCLC) including adenocarcinoma (ADC) and squamous cell carcinoma (SQCC) accounts for approximately 80-90% of all diagnosed lung cancers (1,2). Despite recent advances in understanding complex pathophysiology, uncovering predictive/prognostic markers, and developing novel technologies to aid diagnosis/treatment decision, NSCLC is still the major determinant of overall cancer mortality (3-7). The 5-year survival rate was 15% across all stages of the disease-ranging from 2.8% for patients with distant metastases to nearly 50% for those presenting with local disease. Because NSCLC is a heterogeneous disease entity, the accurate stratification of the patients with high risk of developing recurrence or distant metastasis would improve final prognosis.

While the brain is a major site of relapse contributing to the unfavorable prognosis of the NSCLC, the relevant molecular mechanisms are largely unknown. Lung ADC is known to establish distant macrometastasis within months of initial diagnosis (8-10). This short latency implies that metastatic competence would result from early oncogenic events that drive primary tumor growth rather than late-arising, rare genomic alterations specific for metastasis (11). Therefore, defining consistent chromosomal changes in the primary NSCLC could help to identify metastasis-specific signatures. Molecular inversion probe (MIP) technology is a high-throughput genotyping capable of providing copy number measurements with high precision and specificity, proven to be a valid array for analyzing copy number in formaldehyde-fixed and paraffin-embedded (FFPE) samples (12-14). In this study, to delineate prognostic markers to stratify NSCLC patients at higher risk for developing brain metastasis (BM) and novel therapeutic candidates targeting BM, the patterns of copy number alterations (CNA) in primary lung NSCLC FFPE samples were analyzed by MIP technology.

Materials and methods

Pathology samples from NSCLS patients with brain metastases. Our study was reviewed and approved by the Institutional Review Board of the Samsung Medical Center (Seoul, Korea). All pathology samples and clinical data were obtained with written informed consent according to the institutional regulations. In the pathology database of the Samsung Medical Center (1994.12-2010.7), 160 NSCLC brain metastasis patients were identified. From them, 36 cases were selected; exclusion criteria, the biopsy of primary lung cancer without brain metastasis sample; inclusion criteria, harbouring visible tumors. A total of 37 formaldehyde-fixed and paraffin-embedded (FFPE) pathology samples were analyzed by this MIP analysis including 3 normal lung and 5 brain controls as follows: 20 ADCs of 8 paired cases (lung cancer and BM derived from same patient) and 4 only lung cancer cases; 9 SQCCs of 3 paired cases and 3 only lung cancers. Normal lung and brain tissues were obtained from the patients with other benign diseases.

All NSCLC patients were classified according to the standard World Health Organization (WHO) histological typing of lung carcinomas and the TNM (tumor-node-metastasis) staging system of the International Union Against Cancer (UICC). Clinicopathological data including age, gender, tumor stage and treatment history were obtained by a review of the medical records. Sites of distant metastasis and disease recurrence after treatment were traced by serial computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET). BM was defined as synchronous when metastasis was detected within 3 months of the initial diagnosis of primary tumor. The others were defined as metachronous.

Isolation of genomic DNA. Genomic DNA (gDNA) was extracted from three 5-μm-thick sections per FFPE block for each pathology sample. Deparaffinization and RNA extraction/purification was performed using a QIAamp DNA FFPE\Tissue kit (Qiagen) according the proposed protocol. The only change to the standard protocol was to increase the proteinase K digestion time (overnight). Extracted gDNA was stored at -20˚C, and DNA quantity was analyzed by a Quant-iT Picogreen dsDNA kit (Invitrogen).

Molecular inversion probe (MIP) assay and data analysis. The MIP assay was performed using the OncoScan™ FFPE Express 330K MIP platform (Affymetrix). The MIP assay was performed as described previously (12,15). Briefly, gDNA samples (75 ng per each sample) were annealed with the 24,037 MIP probes (200 amol/μl per probe) in a 384-well plate on ice at 20˚C for 4 minutes (min), at 95˚C for 5 min, and then at 58˚C overnight. The mixtures were circularized with the addition of 4 μl of the appropriate nucleotide at 58˚C for 10 min (gap-fill ligation). Un-circularized MIP probes and gDNA were eliminated by addition of 4 μl of exonucleases (37˚C for 15 min followed by heat inactivation). The circularized probes were linearized by restriction enzyme digest at 37˚C for 15 min, and then amplified using a universal primer for 18 cycles [95˚C for 20 seconds (sec), 64˚C for 40 sec, and 72˚C for 10 sec]. For the labeling reaction, the amplified products were further amplified with the label primers for 10 cycles. The MIP polymerase chain reaction (PCR) products were mixed with hybridization cocktail, denatured, and hybridized to 30K Universal Tag Arrays (Affymetrix) at 39˚C for 16 h with two arrays for each allele. The hybridized arrays were washed on a standard Affymetrix fluidic station and stained with streptavidin-phycoerythrin (5 ng/ml, Invitrogen).

Copy number estimation was obtained from the barcode hybridization signals as described previously (12). The copy number changes of the NSCLCs were analyzed compared with the copy number of the normal non-neoplastic lung and brain samples. Data analysis including copy sum and copy contrast computation, allele ratio, sample normalization, data smoothing was performed by Nexus copy number analytics Version 5.1 Software (Biodiscovery), using algorithm SNP-FASST2 with sensitivity $p<0.05$.

Results

Detailed clinicopathological data of the 18 NSCLC patients with BM (12 ADCs and 6 SQCCs) are summarized in Table I. The median age of the NSCLC patients at the initial diagnosis of the primary cancers was 55 years (range 33-72 years). Synchronous (metastasis within 3 months of the initial diagnosis of primary NSCLC) and metachronous (metastasis after 3 months of the initial diagnosis of primary NSCLC) BM were 6 (33%) and 12 cases (67%), respectively. Median time between initial diagnosis of primary tumors and onset of overall BM was 257.5 days (8.6 months). The median lag (range) till BM for synchronous tumors was 0 (0-88) while for metachronous tumors the lag was 545 (180-2190) days.

Specific copy number alterations depending on distinct histologic-subtypes of non-small cell lung cancer. In this study, chromosomal aberration of 18 NSCLC lung samples (12 ADCs and 6 SQCCs) and 3 normal lung controls was analyzed by the MIP technology to identify chromosomal aberrations involved in the distinct pathogenesis. Common genomic aberrations were defined as continuous genomic regions where the average signal in tumors consistently and significantly differed from that of normal lung samples. Since there is growing evidence that lung ADC and SQCC have distinct oncogenic mutations and divergent therapeutic responses (16,17), 12 ADCs and 6 SQCCs were analyzed separately. A full view of the frequently detected significant chromosomal aberrations is presented in Fig. 1. The most frequent aberrations identified in primary ADCs (>40% of cases) were 3q, 5p, 5q, 6p, 8q, 9p, 11p, 15q and 17q for copy number gains and 10q and 22q for losses (Table II). On the other hand, primary SQCCs revealed frequent copy number gains in 4q and 12q and copy number loss in 9q (Table II). Previously, whole-genome array comparative genomic hybridization (aCGH) identified significantly different chromosomal signatures between two subtypes of the NSCLC (18). Compared with the previous results using different experimental techniques, similar patterns of CNAs were observed in this study, confirming the validity of the MIP technology (19-22). However, several studies demonstrated additional CNAs including gains of 1q and losses of 9q and 10p in ADC and gains of 3q, 7p, 12p and 20q as well as losses of 2q, 3p, 16p and 17p in SQCC (21,23-25).

Continuous somatic evolution eventually giving rise to overt metastasis revealed the metastasis-specific related genes that

Table I. Cliniopathological features of 18 non-small cell lung cancer (NSCLC) cases studied.

Sample ID	Gender/ age	Pattern of metastasis	Subtype	Treatment for PT	Treatment for BM	Extracranial metastasis	Interval between PT and BM (days)	OS[a] (days)	PFS[b] (days)	KRAS mutation	EGFR mutation
1	M/62	S	ADC	Lobectomy, RT	TR, RT, GKRS (2)	None	0	496	110	G12V	wt
2	M/66	S		Lobectomy, RT	TR, RT	None	0	1098	1098	G12D	wt
3	F/55	S		Lobectomy, RT	TR, RT	None	0	1590	1590	wt	L858R
4	M/33	S		Neoadjuvant CCRT, lobectomy	TR (2), RT, GKRS (4)	None	88	670	70	wt	E746-A750 del
5	F/55	M		Lobectomy, CCRT	TR, RT	None	2190	99	-	wt	wt
6	F/66	M		Lobectomy, RT	TR, RT	None	1230	933	271	wt	E746-A750 del
7	F/55	M		Lobectomy, RT	TR	Adrenal gland, liver	1240	30	-	wt	E746-A750 del
8	F/46	M		Lobectomy, CCRT	TR, GKRS, RT	T-spine	780	32	-	wt	wt
9	M/64	M		Neoadjuvant CCRT, lobectomy	TR, GKRS	None	1110	922	-	wt	wt
10	M/51	M		Lobectomy, RT	TR, RT	None	210	254	-	wt	wt
11	M/45	M		Lobectomy	GKRS (2), TR	None	310	922	-	NA	NA
12	M/56	M		Lobectomy, CCRT	TR, RT, GKRS	None	830	405	-	NA	NA
13	M/67	S	SQCC	Lobectomy	TR	None	0	24	-	wt	wt
14	M/54	S		Lobectomy	TR (3), GKRS (4)	None	0	2633	1607	wt	wt
15	F/47	M		Lobectomy, CTx.	TR, RT	None	180	69	-	wt	wt
16	M/72	M		Lobectomy, CTx.	TR, RT	None	310	1210	-	wt	wt
16	M/51	M		Lobectomy, CCRT	TR (2), RT, GKRS (2)	None	210	470	390	wt	wt
17	F/52	M		Lobectomy, CTx.	TR, GKS (2), RT	None	305	136	-	wt	wt

S, synchronous; M, metachronous; PT, primary tumor; BM, brain metastasis; OS, overall survival; PFS, progression-free survival; aber, aberration; ADC, adenocarcinoma; SQCC, squamous cell carcinoma; CTx., chemotherapy; RT, radiation therapy; CCRT, concomitant chemo-radiation therapy; TR, tumor removal (times); GKRS, gamma knife radiosurgery (times); wt, wild-type; del, deletion; NA, not analyzed. [a]OS, overall survival periods from the diagnosis of brain metastasis. [b]PFS, progression-free survival periods from the diagnosis of brain metastasis.

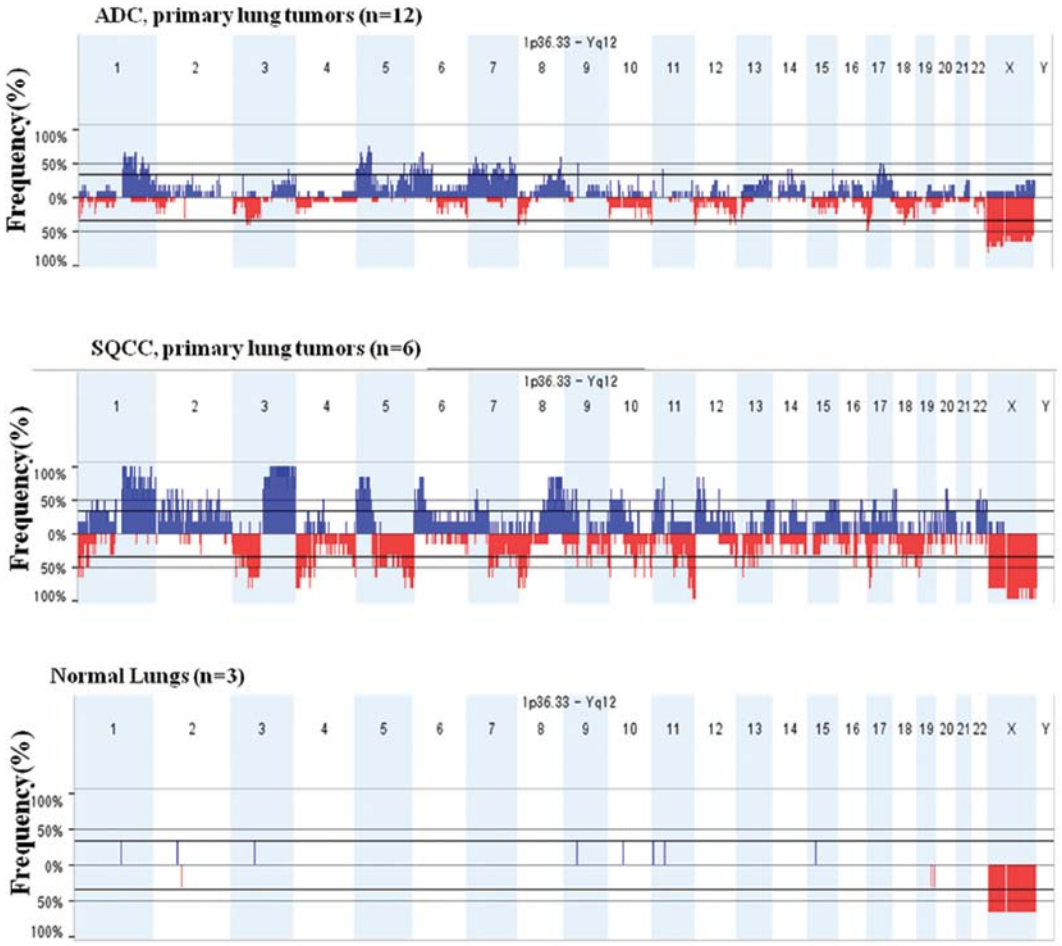

Figure 1. Copy number gains (blue) and losses (red) detected by MIP array in primary lung adenocarcinomas (ADCs, n=12) and squamous cell carcinomas (SQCCs, n=6). Frequencies of genome copy number gains and losses were plotted as a function of genome location with chromosomes 1pter to the left and chromosomes 22qter and X to the right. *ADC, adenocarcinoma; SQCC, squamous cell carcinoma.

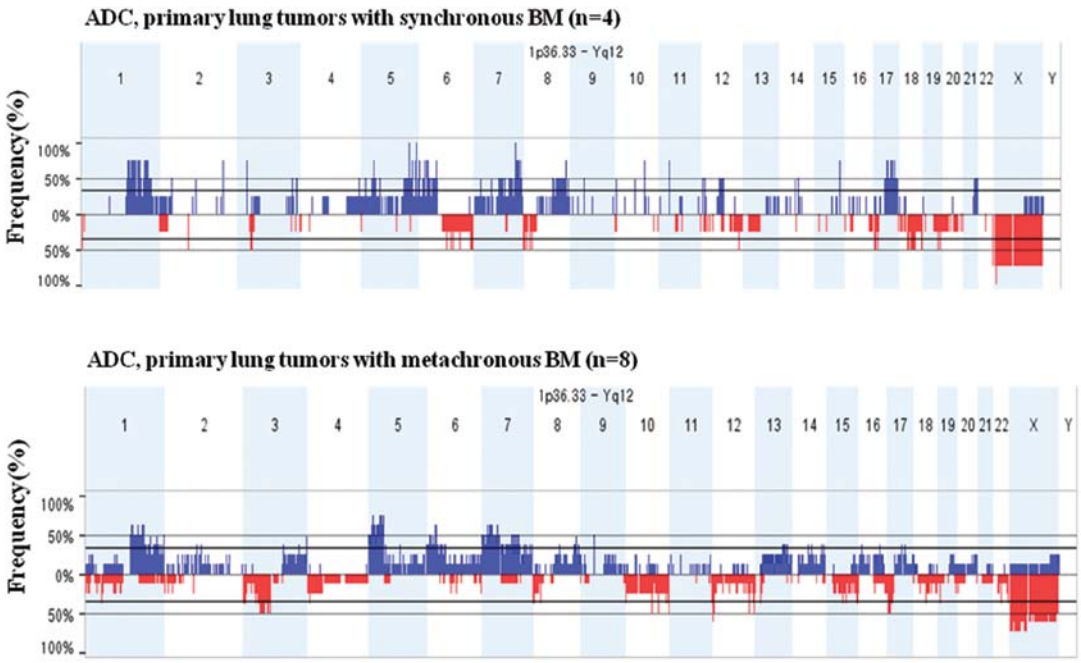

Figure 2. Differential chromosomal abnormalities among primary lung ADCs derived from the patients with synchronous (n=4) and metachronous (n=8) brain metastasis analyzed by MIP array. Frequencies of genome copy number gains and losses were plotted as a function of genome location with chromosomes 1pter to the left and chromosomes 22qter and X to the right. Blue and red columns indicate frequencies of tumors showing copy number gains and losses, respectively. *ADC, adenocarcinoma; BM, brain metastasis.

Table II. Frequent regions of adenocarcinoma-specific and squamous cell carcinoma-specific copy number alteration.

NSCLC subtype	Chromosomal aberration	Region (start site-end site)	Cytoband location	Frequency %	P-value
Adenocarcinoma	CN gain	chr3:173,271,977-173,427,105	q26.31	41.7	0.011
(n=12)	CN gain	chr5:40,349,188-40,886,467	p13.1	75	0.018
	CN gain	chr5:150,438,972-150,523,583	q33.1	50	0.001
	CN gain	chr5:172,100,206-172,250,021	q35.1-q35.2	50	0.001
	CN gain	chr6:26,128,335-26,352,388	p22.1	66.7	0.004
	CN gain	chr8:134,026,676-134,089,398	q24.22	58.3	0
	CN gain	chr9:43,997,314-44,825,192	p11.2	50	0
	CN loss	chr10:133,727,624-135,374,737	q26.3	41.7	0.026
	CN gain	chr11:35,117,949-35,240,617	p13	41.7	0
	CN gain	chr15:83,849,162-83,995,470	q25.3	41.7	0
	CN gain	chr17:45,586,978-45,621,111	q21.33	50	0.015
	CN gain	chr17:55,139,289-55,290,700	q23.1	50	0.015
	CN loss	chr22:47,207,744-47,732,937	q13.32	50	0
Squamous cell	CN gain	chr4:87,398,382-87,491,412	q21.3	50	0.028
carcinoma (n=6)	CN loss	chr9:139,183,759-140,273,252	q34.3	66.7	0.042
	CN gain	chr12:74,190,395-74,668,717	q21.2	66.7	0.026

[a]Regions were included if present in >50% of total adenocarcinoma-specific cases.

Table III. The comparison of recurrent copy number alterations in primary lung adenocarcinomas derived from the patients with synchronous (n=4) and metachronous (n=8) brain metastasis.

Event	Chromosome cytoband	Start	End	Region length	Genes	Frequency % synchronous BM (Total cases = 8)	Frequency % BM metachronous (Total cases = 4)	P-value
Gain	5q35.1	172008530	172100206	91,676	NeurL1B	100	12.5	0.01
	5q35.2	172250021	172277415	27394	ERGIC1	100	12.5	0.01
	10q23.31	90682710	90735753	53043	ACTA2	75	0	0.018
	10q23.31	90749267	90751025	1758	FAS	75	0	0.018
	17q23.3	59424619	59455000	30381	C17orf72, ICAM2	100	0	0.002
	17q24.1	60155862	60203841	47979	LOC146880	100	12.5	0.01
	17q24.1	60635140	60665382	30242	RGS9	75	0	0.018

[a]BM, brain metastasis.

mediate or impede metastatic progression without affecting primary malignancy (26). When patterns of CNAs in 11 BM (8 ADCs and 3 SQCCs) were compared with those of corresponding primary lung tumors, BM were found to carry the majority of genetic alterations present in the corresponding primary tumors (data not shown). However, in ADCs, BM harbored specific CNAs; new 11p and 15q gain (data not shown). In SQCCs, no BM specific CNAs were detected, likely because of the small number (n=3) of the SQCC cases. Therefore, BM specific CNAs were further analyzed in the ADCs.

Genetic signatures associated with early brain metastasis of lung adenocarcinoma. Although comparing the genetic alterations between primary and metastatic tumor would have functional implications, BM-associated genetic signatures in the primary lung ADC would have been more clinically relevant. Lung ADC is characterized by the early development of BM and the incidence of BM based on autopsy findings was as high as 50% in patients with lung ADC (8,27). Given that BM significantly worsens prognosis of lung ADC patients, lung ADC patients who are likely to develop BM need adjuvant treatments for BM. Primary lung ADCs with early development of BM (synchronous) would contain more CNAs predictive of metastatic potential or aggressive transformation. To uncover complex genetic 'signatures' that can predict the risk of BM, the copy number changes of 4 lung ADCs with

synchronous BM were compared with those of 8 lung ADCs with metachronous BM (Table III and Fig. 2). Amplification in 5q35.1-2, 10q23.31, 17q23.3-24.1 and 17q24.1 was detected in 100, 75, 100 and 75% of the cases with synchronous BM, respectively, significantly more frequent than that of lung ADCs with metachronous BM (Table III). Differentially gained regions between primary ADCs with synchronous and metachronous BM were found to contain putative metastasis promoting genes, NeurL1B, ACTA2, FAS and ICAM2 (Table III).

Discussion

Although the BM affecting up to 25% of NSCLC during their lifetime negatively impacts survival, currently, there are no standard practice measures to reduce BM risk in NSCLC (28,29). Under the hypothesis that the genes residing in amplified or deleted regions in each subtype play an important role in the histology-specific pathogenesis of NSCLC, this study was designed to identify 'meta-signatures' that stratify NSCLC patients at higher risk for BM. The NSCLC harbours several histopathologically and molecularly distinct subtypes including ADC and SQCC. However, important molecular differences between primary lung ADC and SQCC have been identified, suggesting that future targeted therapies need to be histology-specific (8,16,17,30-36). For example, KRAS and epidermal growth factor receptor (EGFR) gene mutations are found almost exclusively in ADCs rather than SQCCs. Therefore, primary lung ADCs were separately analyzed in this study.

Due to increasing incidence and substantial relapse rate, the pattern of CNAs was compared between primary ADCs derived from patients with synchronous and metachronous BM to find brain-specific meta-signature and putative targets associated with BM in ADC subtype. Several putative genes reported to be involved in tumorigenesis of various cancers were demonstrated in our study. For example, Neuralized-1B is the E3 ubiquitin ligase that is required for endocytosis regulating both the receptor and ligand side in Notch signaling (37,38). Relatively frequent deregulation of the Notch pathway and Notch ligand Jagged2/miR-200-dependent pathway in NSCLC indicates the significance and mechanisms underpinning Notch pathway activation in lung cancer (39-41). In addition, Fas signalling exhibits tumor-promoting effects by increasing proliferation and invasiveness (42-46). Fas can promote lung cancer growth by recruiting MDSC via cancer cell-derived PGE2 and signal for cell invasion via the glycogen synthase kinase 3β pathway (47,48). Human smooth muscle α-actin (SMA/ACTA2) has been used as one of mesenchymal cell-specific markers showing the epithelial-to-myofibroblast transition involving actin-skeleton remodeling and myogenic reprogramming. The appearance of dot-like α-SMA staining in cytokeratin positive cells may indicate the initial phase of the epithelial to mesenchymal transition (EMT) (49-54). Finally, a recent study reported ICAM2 as a mediator for a survival signal sufficient to block apoptosis by activation of the PI3K/AKT pathway (55). As tumor progression and response to treatment are determined by numerous co-dependent prognostic factors, a multi-genetic approach to determining the optimal treatment for individual patients is more likely to be successful rather than a single prognostic biomarker.

Evidence that highly metastatic clones from primary tumor had a higher rate of genetic mutability than non-metastatic clones from the same tumor provided an early link between metastasis and intrinsic genetic instability such as mutations and chromosomal rearrangements (56). Although several high-throughput technologies have been developed to detect genomic CNAs in a variety of cancers until recently, high sensitivity to detect single copy number changes and the ability to test FFPE samples in which DNA is known to be degraded to different degrees is important (12). Analyzing FFPE samples allows the utilization of the vast majority of cancer samples since most cancer tissue samples are available as FFPE. For our FFPE NSCLC samples, the MIP technology was used, which has been validated to be able to substitute aCGH (12-15,57,58). Because MIP uses probes that have a genomic footprint of ~40 bp, this high density platform only requires small amounts of DNA and, consequently, is well-suited for the analysis of degraded DNA in FFPE tissues (59). The results of this study using the MIP technique showed differential genetic alteration between lung ADCs and SQCCs, correlating well with previous studies using aCGH. The similarities support the validity of the technique and the results from it. However, due to inadequate amount of extracted gDNA, quantitative-real-time-PCR (qRT-PCR) to validate copy number alterations identified by the MIP array could not be performed in our study.

In this study, we identified several genes associated with early BM of lung ADCs. Although our data suggested a new aspect of genomic alterations associated with BM of NSCLC, further biological studies to validate the role of identified candidates in brain-specific metastasis and testing the predictive power of our meta-signature in larger NSCLC samples would be required. In spite of several limitations of our study including very small number of NSCLC samples and absence of validation studies such as qRT-PCR, these genomic signatures may help to generate useful markers to refine prognosis and guide therapeutic decisions for improvement of prognosis and quality of life for patients with NSCLC.

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