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Original Article

EphA2, a possible target of miR-200a, functions through the AKT2 pathway in human lung carcinoma

Hiroyoshi Tsubochi[1], Kentaro Minegishi[1], Akiteru Goto[2], Ritsuko Nakamura[3], Daisuke Matsubara[4], Yoh Dobashi[5,6]

Departments of [1]Thoracic Surgery and [5]Pathology, Saitama Medical Center, Jichi Medical University, Omiya, Saitama, Japan; [2]Department of Cellular and Organ Pathology, Akita University School of Medicine, Japan; [3]Department of Molecular and Cellular Pathology, Graduate School of Medical Sciences, Kanazawa University, Kanazawa, Ishikawa, Japan; [4]Department of Integrative Pathology, Jichi Medical University, Shimotsuke, Tochigi, Japan; [6]Department of Pathology, International University of Health and Welfare Hospital, Nasushiobara, Tochigi, Japan

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Abstract: We previously reported that miR-200a was highly up-regulated in lung carcinoma, exhibiting a copy number increase (CNI) of the AKT2 gene (AKT2+ group) in defined subsets, i.e., adenocarcinoma and early stages of carcinoma (pStage I/II). In this study, we searched possible targets of miR-200a in these subsets by IHC analyses focusing on the expression of known target proteins of miR-200a: beta-catenin, EphA2, ZEB1, PTEN, and YAP-1, as well as E-cadherin, the expression of which is suppressed by ZEB1. Among those 6 proteins, when all 38 cases of surgically resected specimens were analyzed as a whole, IHC score of ZEB1 was inversely (ρ=-.417) and E-cadherin was positively (ρ=.345) correlated with miR-200a expression. However, only EphA2 was inversely correlated with the expression of miR-200a in adenocarcinoma (ρ=-.496) and in pStage I/II group (ρ=-.547), while no correlation was seen in non-adenocarcinoma, squamous cell carcinoma, or pStage III carcinoma. Furthermore, by comparison of 3 groups categorized according to the AKT gene increase, only EphA2 was down-regulated to a statistically significant level in the AKT2+ group in both adenocarcinoma (p=.0447) and pStage I/II carcinoma (p=.0458). These results suggest that in lung carcinomas, higher Akt activation caused by increased AKT2 gene copy number leads to the upregulation of miR-200a, which exerts its function as a suppressor of EphA2 in adenocarcinoma and the early stages of carcinomas.

Keywords: Lung cancer, AKT, amplification, miR-200a, ZEB1, EphA2

Introduction

Lung cancer is the most common cause of cancer mortality worldwide [1]. Even in recent decades, its 5-year survival rate remains 13%-16%, which is due to high rates of local recurrence and/or distant metastasis [1]. Over the past few decades, a number of biomarkers have been identified that help to guide the diagnosis and evaluation of both the stage and prognosis of this cancer. Moreover, an improvement in overall survival has been achieved by the integration of targeted treatments. These include the tyrosine kinase inhibitors which interfere with the epidermal growth factor receptor, anaplastic lymphoma kinase, BRAF, and others [2]. However, there remains a high medical need for novel, personalized medicine therapies.

MicroRNAs (miRs) are a class of small non-coding RNAs (approximately 17 to 28 nucleotides) that are involved in the post-transcriptional regulation of gene expression [3]. miRs function primarily to reduce mRNA levels, and to a lesser extent by suppressing translation [3, 4]. To date, numerous studies have identified dysregulated miRs associated with cancer, (called 'onco-miRs'), and these have been found to play important roles in carcinogenesis, metastasis, and the progression of many types of cancer, including lung carcinoma [3].

We previously examined miRNA expression profiles in lung carcinomas by microarray analysis,

Table 1. Patients' characteristics

		AKTd/d[1)]	AKT1+[2)]	C. AKT2+[3)]
Cases		13	9	16
pT	T1	2	4	4
	T2	6	2	5
	T3	4	2	6
	T4	1	1	1
pN	N0	4	7	6
	N1	4	1	3
	N2	5	1	7
pStage	I	2	4	4
	II	6	3	5
	III	5	2	7
Histology	AC[4)]	6	5	6
	SCC[5)]	4	2	6
	LCC[6)]	1	1	0
	SmCC[7)]	2	1	4

Abbreviations. 1) cases harboring both AKTs genes in disomy; 2) cases with only AKT1 increase by amplification or high-level polysomy, and with AKT2 in disomy; 3) cases with only AKT2 increase; 4) AC, adenocarcinoma; 5) SCC, squamous cell carcinoma; 6) LCC, large cell carcinoma; 7) SmCC, small cell carcinoma.

and detailed statistical analysis of the data has revealed a unique miRNA expression pattern associated with increased AKT1 and AKT2 gene copy number [5]. First, the miR-200 family, i.e. miRs-141, -200a, -200b, and -200c were all found to be up-regulated in groups of cancers harboring copy number increases (CNI) of AKT1 (AKT1+ group) or AKT2 (AKT2+ group). Second, in adenocarcinoma (AC) and early-stage carcinoma (pStage I/II), expression of miR-200a was higher in the AKT2+ group compared with the AKT1+ group or cases exhibiting disomy of both AKT1 and AKT2, with a statistical significance of P=0.0334 and P=0.0239 for AC and pStage I/II, respectively. Therefore, AKT2 gene copy number is associated with miR-200a expression in a histology- or stage-specific manner. Several targets of mir-200a have been described and representative ones involved in cancer include beta-catenin (β-catenin) [6], the Eph receptor A2 (EphA2) [7], Zinc finger E-box-binding homeobox 1 (ZEB1) [6], phosphatase and tensin homolog 10 (PTEN) [4] and Yes-associated protein 1 (YAP-1) [8]. In the present study, we performed immunohistochemical analysis of these candidate downstream targets to better understand the pathway leading from AKT2 through miR-200a that contributes to the pathology of lung carcinomas exhibiting AKT2 CNIs.

Materials and methods

Cases and classification

We analyzed the same 38 cases of lung carcinoma that had been used previously to investigate the profile of miR expression [5]. These cases had been obtained in the Jichi Medical University Hospital following institutional review board approval (Approval No. 17-45) and written informed consent was obtained from each patient. These cases were stratified into three groups by AKT gene status [5]. The first group consisted of 13 cases in which both AKTs genes exhibited disomy (termed AKTd/d) and included 6 cases of AC, 4 of squamous cell carcinoma [SCC], 1 of large cell carcinoma [LCC] and 2 cases of small cell carcinoma [SmCC]). The second group consisted of 9 cases where AKT1 gene number was increased by amplification or high-level polysomy, while the AKT2 gene exhibited disomy (named AKT1+) and included 5 cases of AC, 2 of SCC, 1 case each of LC and SmCC. The third group consisted of 16 cases where AKT2 gene number was increased, while the AKT1 gene exhibited disomy (named AKT2+) and included 6 cases of AC, 6 of SCC and 4 cases of SmCC. Further details of these cases are presented in **Table 1**.

Immunohistochemistry (IHC)

We performed IHC-based analyses to ask what effect upregulated miR-200a has on particular target proteins. Sections of 3.5 μm thickness were stained by Autostainer Link 48 (Agilent, Santa Clara, CA) using primary antibodies against proteins that have been described as being regulated by miR-200a, including β-catenin (#IR702, mouse monoclonal, clone M35-39, Agilent) at 1:300, EphA2 (#MA5-15284, mouse monoclonal, clone 1B3C7, ThermoFisher Scientific [Invitrogen], Waltham, MA) at 1:600, ZEB1 (HPA027524, polyclonal, Sigma-Aldrich, St. Louis, MO, USA) at 1:500, PTEN (#51-2400, rabbit polyclonal, ThermoFisher Scientific [Invitrogen]) at 1:200 and YAP-1 (ab52771, rabbit monoclonal, clone EP1674Y, Abcam, Cambridge, UK) at 1:200, as well as E-cadherin (mouse monoclonal, clone 36, BD Biosciences, Franklin Lakes, NJ, USA) at a dilution of 1:400. The sensitivity and the specificity of these antibodies were previously validated by us [5] and others [9-12]. Heat-induced epitope retrieval was per-

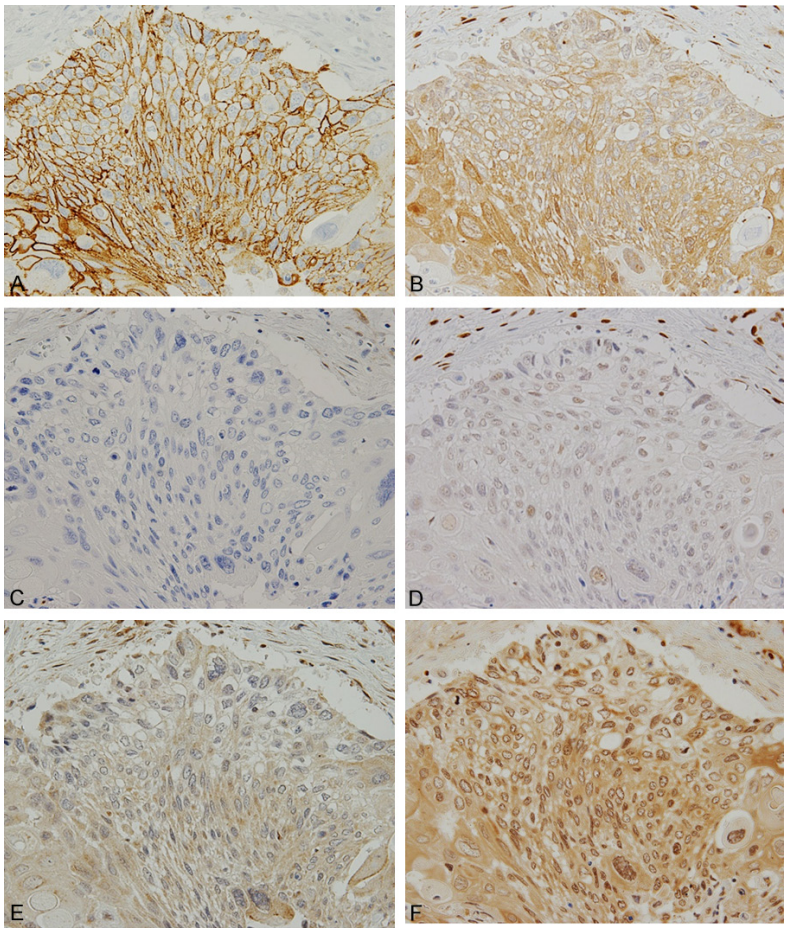

Figure 1. Immunohistochemical staining for E-cadherin, β-catenin, EphA2, ZEB1, YAP-1 and PTEN in a case of squamous cell carcinoma, showing a higher level of miR-200a expression (4.55). A. E-cadherin expression was observed on the membrane of tumor cells (immunohistochemical score, 6). B. β-catenin was also observed on the membrane (score 4). C-E. No expression of EphA2 and negligible level of expression in ZEB-1 and PTEN, respectively (score 0). F. YAP-1 was strongly expressed in the cytoplasm and nucleus (score 6). A-F. Original magnification, ×200.

formed using PT Link (#PT100/PT101, Agilent) with EnVision™ FLEX Target Retrieval Solution, Low pH (pH. 6.0, Code S2031) for ZEB1, PTEN, Eph2 and E-cadherin, and High pH (pH9.0, S2367) for β-catenin and YAP-1. Visualization was performed with a CSAII kit (Catalyzed Signal Amplification System 2, Agilent). Staining was evaluated by three observers (HT, DM and YD). IHC score was determined semi-quantitatively by multiplication of the "positive fraction" with the "intensity-score" according to the following tier system: i) "positive fraction" was categorized as 0, no staining; 1+, ≤10%; 2+, 10%<, ≤50%; 3+, 50%<, ii) "intensity score" was as 0, no staining; 1, weaker than, or the same as that in non-neoplastic cells; 2, more intense than the staining in non-neoplastic cells [5, 9, 13]. IHC score of more than 1 which is, therefore, intensity score of more than 1, was defined as "positive".

Statistics

We analyzed the IHC data to identify proteins i) that showed significant positive or inverse correlations with miR200a levels, and ii) up-. or downregulated in AKT-2+ groups compared with AKT1+ or d/d groups to a statistically significant level. Accordance among observers in the evaluation of IHC results was analyzed by kappa (κ) statistics as follows: 0, no agreement; 0<, ≤0.20, slight agreement; 0.20<, ≤0.40, fair agreement; 0.40<, ≤0.60, moderate agreement; 0.60<, ≤0.80, substantial agreement; 0.8<, ≤1.00, almost perfect agreement. Other statistical analysis was performed with JMP software package (version 11, SAS Institute Inc., Cary, NC). Correlation between the expression of miR-200a and proteins was analyzed by the Spearman rank correlation test and results were categorized as follows: no correlation, coefficient (ρ)=0; equivocal, |ρ|≤0.2; low, 0.2<|ρ|≤0.4; substantial, 0.4<|ρ|≤0.7; high, 0.7<|ρ|<1.0; complete, ρ=1.0. Differences in the levels of IHC expression represented by IHC scores among the groups were analyzed by Fisher's PSLD test.

Results

IHC

As shown in **Figures 1** and **2**, and summarized in **Table 2**, expression of these proteins was clearly observed. Inter-observer agreement in the evaluation of staining results ranged from "substantial agreement" to "almost perfect

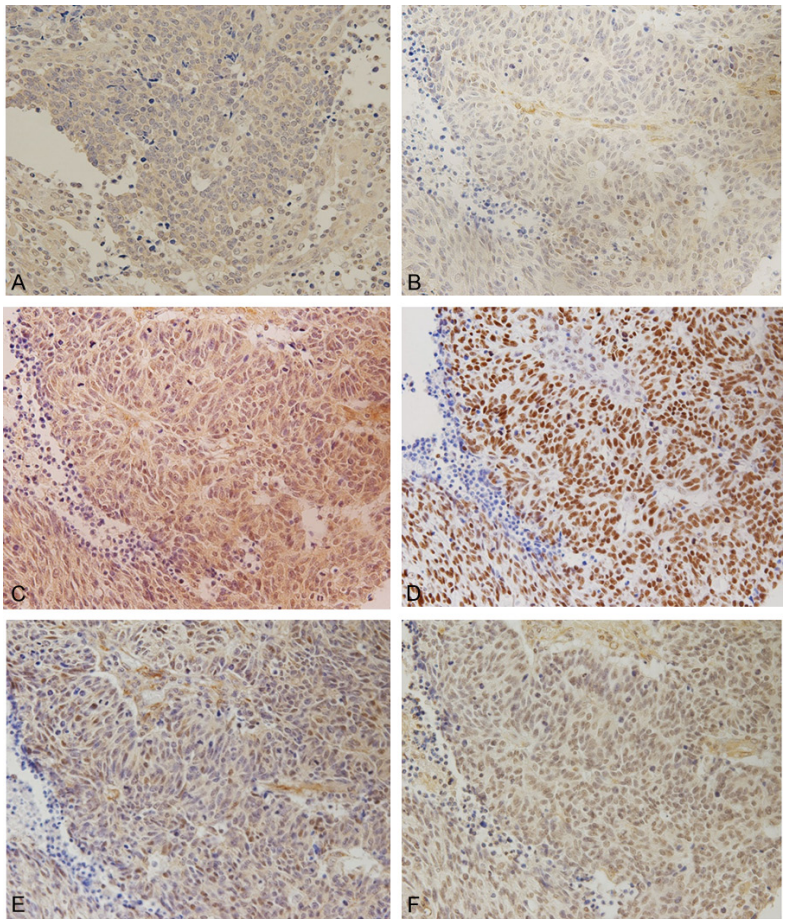

Figure 2. Immunohistochemical staining for a case of large cell carcinoma showing a lower level of miR-200a expression (0.29). A, B. E-cadherin and β-catenin, respectively, were not significantly expressed (immunohistochemical score 0). C. EphA2 expression was observed on the membrane and in the cytoplasm of tumor cells (score, 6). D. ZEB-1 was observed in the nucleus (score 4). E. PTEN was focally expressed in the cytoplasm (score 2) F. YAP-1 was expressed in the cytoplasm and the nucleus (score 6). A-F. Original magnification, ×200.

agreement" (ρ; E-cadherin=.781; β-catenin =.782; Eph-A2=.860; ZEB1=.897; PTEN=.816; YAP-1=.893). The staining results are detailed below.

E-cadherin: In non-neoplastic tissues, clear membranous staining was observed in the bronchial and in alveolar epithelial cells. In tumors, positive staining was observed on the membrane in all but one case of LCC and two cases of SmCC (35/38 cases, 92.1%). The IHC score varied depending on the histologic type: 64.7% of the positive cases in AC (11/17) and 50.0% of SCC (6/12 cases) showed a high score, such as ≥4+, compared to 20% (1/5 cases) in SmCC, and none in LCC.

β-catenin: In non-neoplastic tissues, membranous staining was observed in the bronchial and, occasionally, alveolar epithelial cells. This pattern of staining positivity in tumors was observed in 30/38 cases (78.9%) and was the highest in AC (94.1%, 16/17 cases), followed by SCC (83.3%%, 10/12 cases). IHC score was the highest in AC, where 50.0% of the positive cases (8/16) showed a high score, ≥4+, followed by SCC (30.0% of 3/10 cases), but none in LCC or SmCC.

EphA2: In non-neoplastic tissues, no significant staining was observed. In tumors, positive staining was observed on the membrane as well as the cytoplasm in 30 cases (30/38 cases, 78.9 %). High IHC score (≥4+) was observed for all cases of LCC (2/2 cases) and SmCC (5/5 cases), but only 41.7% (5/12) in AC and 27.3% (3/11 cases) in SCC.

ZEB1: In the non-neoplastic tissue, the nuclei of bronchial cells, alveolar epithelial cells, lymphocytes, and endothelial cells occasionally showed positive staining, but otherwise this was not significant. In tumors, staining positivity was observed in 13 cases (13/38 cases, 34.2%). IHC score was highest in SmCC, where 80.0% (4/5 cases) of the positive cases stained 4+, whereas only 50.0% (2/4) of the positive cases in SCC and 33.3% (1/3 case) of AC showed ≥4+ staining.

PTEN: In non-neoplastic tissues, weak staining was observed in the cytoplasm of bronchial cells, alveolar macrophages, and the nuclei of lymphocytes and endothelial cells. In tumors, staining positivity was observed in 25/38 cases (65.8%). All (6/6 cases) of the positive cases in SmCC had an IHC score of ≥4+, but 16.7% (1/6) in SCC, and none in LCC.

YAP-1: In non-neoplastic tissues, weak staining was occasionally observed in the cytoplasm and focally in the nuclei of bronchial cells, alve-

Table 2. Results of immunohistochemical staining

	AC[1]				SCC[2]				LCC[3]				SmCC[4]			
	IHC score[5]				IHC score				IHC score				IHC score			
	0	2	4	6	0	2	4	6	0	2	4	6	0	2	4	6
Target proteins																
E-cadherin	0	6	6	5	0	6	4	2	1	1	0	0	2	4	1	0
β-catenin	1	8	5	3	2	7	3	0	1	1	0	0	4	3	0	0
EphA2	5	7	3	2	1	8	1	2	0	0	1	1	2	0	1	4
ZEB-1	14	2	1	0	8	2	1	1	1	0	1	0	2	1	2	2
PTEN	5	3	4	5	6	5	0	1	1	1	0	0	1	0	2	4
YAP-1	6	5	1	5	3	1	2	6	0	0	0	2	3	2	0	2

Abbreviations. 1) AC, adenocarcinoma; 2) SCC, squamous cell carcinoma; 3) LCC, large cell carcinoma; 4) SmCC, small cell carcinoma; 5) IHC score, immunohistochemical score. Each value shows the number of cases with respective immunohistochemical scores.

Table 3. Correlation coefficients (ρ) and *p*-values between miR-200a vs. target proteins

	All Cases	AC[1]	p-Stage I/II	SCC[2]	p-Stage III
E-cadherin (ρ)	0.345*	0.344	0.331	0.425	0.382
	P=0.0332	P=0.0691	P=0.0749	P=0.1237	P=0.0555
β-catenin (ρ)	0.303	0.277	0.297	0.337	0.307
	P=0.5496	P=0.2686	P=0.1551	P=0.3398	P=0.8502
EphA2 (ρ)	-0.386	-0.496*	-0.547*	-0.242	-0.333
	P=0.0914	P=0.0470	P=0.0196	P=0.1062	P=0.1201
ZEB-1 (ρ)	-0.417*	-0.405	-0.257	-0.329*	-0.606*
	P=0.0372	P=0.0667	P=0.0677	P=0.0125	P=0.0233
PTEN (ρ)	-0.288	-0.271	-0.201	-0.502	-0.497
	P=0.1762	P=0.0776	P=0.2586	P=0.1556	P=0.0628
YAP1 (ρ)	0.046	0.106	0.064	0.167	0.070
	P=0.7758	P=0.6711	P=0.7632	P=0.6374	P=0.7944

Immunohistochemical scores of respective proteins and miR-200a expression levels were compared by Spearman's rank correlation test. ρ; ρ=0, no correlation; |ρ|<0.2, equivocal; 0.2≤|ρ|<0.4, low correlation; 0.4<|ρ|<0.7, substantial correlation; 0.7<|ρ|<1.0, high correlation; |ρ|=1.0, complete correlation.
Abbreviations. 1) AC, adenocarcinoma; 2) SCC, squamous cell carcinoma.
*Statistically significant.

olar epithelial cells, lymphocytes, and endothelial cells. In tumors, positive staining in the cytoplasm and/or nucleus was observed in 26/38 cases (68.4%). All (2/2) of the positive cases in LCC and 88.9% (8/9 cases) in SCC had an IHC score of ≥4+, but 50.0% (2/4 cases) in SmCC as the lowest frequency.

Correlation between miR-200a and expression of possible target proteins

To analyze the regulation of cancer-related proteins by miR-200a, in particular, to identify *a* possible critical target of miR-200a downstream of increased AKT2 gene number, we analyzed the correlation between the miR-200a levels, which had been evaluated and described previously [5], and the IHC score of its known target proteins both among the total cases and within defined specific subsets (**Table 3**). In all cases examined as a whole, the highest correlation efficient (ρ) by Spearman' rank correlation test was obtained between miR-200a and ZEB-1 with ρ=-.417 (substantial inverse correlation, P=0.0372). Thus, this miR200a/ZEB-1 pathway is functioning. On the other hand, E-cadherin showed a low positive correlation with miR-200a, with ρ=.345 (P=0.0332) in the current study. However, β-catenin (ρ=.303, P=0.5496), EphA2 (ρ=-.386, P=0.0914), PTEN (ρ=-.288, P=0.1762) and YAP-1 (ρ=.046, P=0.7758) showed no significant correlation. When the analysis was restricted to AC cases, a substantial inverse correlation was found only between miR-200a *and* EphA2 (ρ=-.496, P=0.0470). For ZEB-1, the *p* value suggested a "substantial inverse correlation", but this was not significant (ρ=-.405, P=0.0667). For the other 4 proteins, no significant correlation was obtained. In contrast, when the SCC cases were analyzed, a low inverse correlation was obtained only for ZEB-1

Table 4. Comparison of target protein expression (*p*-values)

	AKT(d/d)[1] vs AKT1+[2]	AKT(d/d) vs AKT2+[3]	AKT1+ vs AKT2+
(a) Adenocarcinoma			
E-cadherin	0.6025	0.3039	0.6934
β-catenin	0.593	0.7473	0.8078
EphA2	0.119	0.0372*	0.0447**
ZEB-1	0.1213	0.5906	0.0568
PTEN	0.3882	0.6756	0.2378
YAP-1	0.1302	0.1923	0.7092
(b) Stage I/II			
E-cadherin	0.4723	0.3738	0.113
β-catenin	0.5405	0.9336	0.4612
EphA2	0.1821	0.0392*	0.0458**
ZEB-1	0.2767	0.5889	0.0992
PTEN	0.7185	0.5648	0.3521
YAP-1	0.3328	0.1412	0.6987
(c) All cases			
E-cadherin	0.7409	0.5600	0.8437
β-catenin	0.7573	0.3424	0.5684
EphA2	0.6407	0.0581	0.0745
ZEB-1	0.9837	0.8431	0.8343
PTEN	0.4569	0.8623	0.3369
YAP-1	0.4125	0.2444	0.8204
(d) Stage III			
E-cadherin	0.9191	0.1113	0.1428
β-catenin	0.6988	0.3260	0.6611
EphA2	0.6912	0.2546	0.5697
ZEB-1	0.4695	0.8532	0.3605
PTEN	0.8764	0.5273	0.4850
YAP-1	0.8970	0.9862	0.9027
(e) SCC[4]			
E-cadherin	0.1835	0.8298	0.1250
β-catenin	0.0474	0.0149	0.7331
EphA2	0.9301	0.5041	0.4969
ZEB-1	0.1997	0.3232	0.5897
PTEN	0.1066	0.0956	0.0924
YAP-1	0.8338	0.8022	0.9155

Abbreviations. 1) cases harboring both AKTs genes in disomy; 2) cases with only AKT1 increase by amplification or high-level polysomy, and AKT2 in disomy; 3) cases with only AKT2 increase; 4) SCC, squamous cell carcinoma. *AKTd/d>AKT2+, **AKT1+>AKT2+.

with ρ=-.329 (P=0.0125), while the correlation between mirR-200a and EphA2 as well as the other 4 proteins was not significant (the lowest P=0.1062 in EphA2). When analysis was restricted to the pStage I/II group, a significant ρ value was found between miR-200a and EphA2 (ρ=-.547, P=0.0196), but not with the other 5 proteins. In the pStage III group, a "substantial inverse correlation" was obtained only for ZEB-1 with ρ=-.606 (P=0.0233), but not with the other 5 proteins (the lowest P=0.0555, E-cadherin).

Difference in the expression of target proteins among groups categorized by AKT status

Next, to further confirm those critical gene products that play a central role in the AKT2+ group, we analyzed the difference in expression of each protein among 3 groups: AKTd/d, AKT1+ and AKT2+. By statistical analysis of the IHC scores, a significant difference was found for EphA2 in the AKT2+ group compared to the AKTd/d (P=0.0372) and the AKT1+ groups in AC (P=0.0447), as determined by Fisher's PSLD test (**Table 4**). Similarly, in the pStage I/II group, a significant difference was also found in the AKT2+ group compared to the AKTd/d (P=0.0392) and AKT1+ groups (P=0.0458), namely, in the AKT2+ group, the EphA2 IHC score was significantly lower than in the other two groups, both among the AC cases and those in pStage I/II. However, the EphA2 IHC score was not different between the AKTd/d and AKT1+ groups either among AC cases (P=0.119) or in pStage I/II (P=0.1821). Although a significant inverse correlation was obtained between the expression of miR200a and ZEB1 in all cases as a whole, and the ZEB1 IHC score trended slightly lower in the AKT2+ compared to the AKTd/d and AKT1+ groups, the differences among these three groups within the AC cases (the lowest P=0.0568 in AKT1+ vs AKT2+) and in pStage I/II cases (the lowest P=0.0992, in AKT1+ vs AKT2+) were not significant (**Table 4**). Similarly, E-cadherin scored highest in the AKT2+ group, but the difference among the groups within AC cases (the lowest P=0.3039, AKTd/d vs AKT2+) and in pStage I/II cases (the lowest P=0.1130 in AKT1+ vs AKT2+) was not significant. For β-catenin, PTEN and YAP-1, the difference in expression levels among these three groups either within AC or

pStage I/II categories was not observed to a statistically significant level. To confirm our observation that EphA2 expression was lower in the AKT2+ group in only for the AC and pStage I/II cases, we performed the same analysis on other defined subsets. However, statistically significant differences were not observed for any protein within "all cases", "non-AC", "SCC", or "pStage III" groups, (**Table 4**).

Overall, in the AC and pStage I/II groups, the one target found to be significantly regulated by miR-200a downstream of increased AKT2, was EphA2. Therefore, an axis emanating from upregulated miR-200a due to AKT2 CNIs appears to function, more or less, via EphA2, ZEB1 and E-cadherin, but has the most potent effect on EphA2.

Discussion

Based on our previous study of lung carcinomas showing that miR-200a was upregulated in AC and in the early stages of carcinomas (pStage I/II) harboring CNI of AKT2, we searched for potential targets of miR-200a within defined cancer subgroups. To date, several targets of miR-200a that are involved in carcinogenesis and/or cell proliferation have been described. β-catenin is a cytoskeletal protein localized in the inner part of the membrane and associated with E-cadherin. miR-200a directly interacts with the 3'-UTR of CTNNB1, the gene encoding β-catenin, and thereby suppress β-catenin expression [14]. It is, therefore, a critical effector molecule in the Wnt/β-catenin signal transduction cascade implicated in human cancers. For example, in colorectal cancers, mutations in the Wnt/β-catenin signaling pathway play a predominant role in carcinogenesis [15]. It also plays a role in the epithelial to mesenchymal transition (EMT), a process associated with tumor metastasis in cancer cells, and this has been shown to be due to the aberrant epigenetic silencing of miRNA [6].

EphA2 is a member of the Eph receptor family, the largest family of receptor tyrosine kinases, and has been shown to control cell growth, migration, invasion [7, 16-18]. Indeed, EphA2 is frequently overexpressed and has been correlated with aggressive biological behavior and poor prognosis in glioblastoma, colon, breast and non-small cell lung carcinoma (NSCLC) [16, 17]. In NSCLC, positive immunostaining of EphA2 has been observed in the membrane and cytoplasm in more than 70% of cases, regardless of histological type [19]. Moreover, higher expression of EphA2 is observed in clinically advanced stages of disease, but is negligible in normal lung tissue [16, 18]. Therefore, EphA2 has been proposed as a potential therapeutic target for these kinds of tumors [16-18].

ZEB1 has been recognized as one of the main targets of miR-200a and has been shown to promote tumorigenesis and EMT by suppressing E-cadherin expression [6, 20]. Accordingly, the expression of ZEB1 is lower in epithelial tissues [20] and is negatively correlated with miR-200a expression [21].

PTEN is a well-known suppressor protein interfering with the PI3K-Akt pathway. Transcriptional/post transcriptional alterations in *PTEN*, including epigenetic alteration, have been described in cancers [4]. In human endometrioid carcinoma, *PTEN* was found to be a target of miR-200a, resulting in reduced expression [4]. However, in our present study, we were unable to confirm any involvement of PTEN downstream of AKT2 modulated by miR-200a.

YAP1, located at 11q22, is widely recognized as a critical downstream effector of the Hippo pathway and functions as a transcription cofactor to promote cell growth [22]. When the Hippo pathway is inactivated, cytoplasmic YAP1 is dephosphorylated and accumulates in the nucleus [23] and therefore is often localized in the tumor cell nuclei. This nuclear overexpression of YAP1 has been correlated with poor overall survival in NSCLC [23]. However, its pathobiologic function appears to be quite diverse, and while YAP1 acts as an oncogene in NSCLC, it also appears to function as a tumor suppressor in other kinds of cancers [22].

E-cadherin is abundant in epithelial cells [24] and its expression is not directly regulated by miR-200a, but has been shown to be repressed by the transcription factor, ZEB-1, which is, in turn, negatively regulated by miR-200a [21]. Therefore, E-cadherin is indirectly regulated by miR-200a through ZEB1 and indeed, its expression is negatively correlated with ZEB1 expression [24].

Thus, these 6 proteins can play critical roles in cancer pathology and therefore were examined as potential downstream effector proteins

modulated by AKT2/miR-200a. Our analysis suggested EphA2 as a critical candidate miR-200a target, functioning downstream of increased AKT2 gene in two subsets, although this association was made from a small total number of cases. First, a significant correlation in the expression of miR-200a with E-cadherin and ZEB-1 was observed when all cases were analyzed as a whole. However, when analysis was restricted to AC and pStage I/II cases, we observed a statistically significant inverse correlation only between miR-200a and EphA2. Second, in a comparison of three groups, i.e., AKTd/d, AKT1+ and AKT2+, EphA2 was significantly downregulated in AC and pStage I/II of the AKT2+ group, but not in other subsets, including cases of non-AC, SCC or pStage III tumors. Therefore, we presume that EphA2 is a possible target of miR-200a in these *particular subsets*, and the AKT2/miR200a-dependent pathway functions through EphA2.

miR-200a has been shown to reduce EphA2 expression in breast cancer cells by direct interaction with the EphA2 3'-UTR [7]. In addition to the miR-200a-E-cadhein pathway responsible for epithelial differentiation, the AKT2-miR-200a-EphA2 axis may represent a novel mechanism to maintain a less aggressive cancer phenotype. In the current study, we did not observe morphological evidence of EphA2 function, such as poorer differentiation indicated by EMT, in all of the cases where EphA2 was overexpressed (data not shown), although it has been previously reported that EphA2-overexpressing cells display a mesenchymal-like phenotype, such as downregulation of E-cadherin and upregulation of N-cadherin [25].

Although a miR-200a/EphA2 axis appears to exist, we were unable to explain the mechanism and the reason by which AKT2 regulates this pathway. Akt promotes cell migration and endows cancer cells with invasive properties through the phosphorylation of EphA2 [26]. It is possible that miR-200a functions as a safeguard mechanism suppressing the effect of overexpressed Akt2 until cancers progress into a more advanced and aggressive stage [27].

The role of miRNAs in biological and pathological processes is quite diverse [3] and the same miRNA can function to both promote and inhibit cell proliferation and survival [27]. In addition, EphA2 is regulated by miRNAs other than miR-200a. For example, miR-26a has been demonstrated to upregulate EphA2 in experimental lung injury [28]. EphA2 gene transcription is also regulated by a variety of stimuli, such as estrogen receptor signaling, c-Myc [17] and the Ras-MAPK pathway [17, 25]. MAPK signaling also activates AKT and promotes EphA2 expression and accumulation in tumor cells [29]. Moreover, EphA2 has been described to be involved in the feedback inhibition of K-ras, resulting in the downregulation of MAPK signaling [30]. Thus, these accumulated facts indicate that EphA2 and miR-200a are not solely regulated by each other and that in cancer EphA2 could act both as a tumor-promoting and tumor-inhibiting factor [30]. These complex mutual interactions between AKT and EphA2 make it difficult to unravel the exact pathologic mechanism of the pathway from AKT2 through miR200a to EphA2 in this setting.

Overall, we show that EphA2 may be a target of miR-200a in AC and in pStage I/II cancers that exhibit AKT2 CNI. In this sense, AKT2-miR200a status could be another biomarker which predicts biological behavior imparted by EphA2 expression in human lung carcinomas.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hiroyoshi Tsubochi, Department of Thoracic Surgery, Saitama Medical Center, Jichi Medical University, 1-847, Amanuma, Omiya, Saitama 330-8503, Japan. Tel: +81-48-648-5188; Fax: +81-48-648-5188; E-mail: h-tsubochi@omiya.jichi.ac.jp

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