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ORIGINAL ARTICLE

Increased expression of ephrin A1 in brain arteriovenous malformation: DNA microarray analysis

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Abstract A number of previous studies have revealed the abnormal expression of various angiogenesis-related genes or products in brain arteriovenous malformation (AVM). To understand the molecular process of this disease, we analyzed gene expression profiles in brain AVM. Using a DNA microarray consisting of 17,086 genes, we identified differentially expressed genes in 5 brain AVMs from their draining veins, vessels retaining basic venous architecture. Not many genes were differentially expressed between the AVM nidus and the draining vein. When we applied an absolute cut-off value for normalized $\log_2$ (cy5/cy3 ratio) of 0.4, 19 genes were selected. Genes such as SOX8, TRIM2, FENA1 (ephrin A1), and AQP4 were upregulated, and genes such as I_1000105, KRT18, IGFBP7, EMILIN-2, and KRT14 were downregulated. Genes relating to angiogenesis, such as vascular endothelial growth factor and angiopoietin and other members of the ephrin family, were not differentiated. Among differentially expressed genes detected in this analysis, we focused on ephrin A1, a gene related to embryogenesis and angiogenesis. The expression of ephrin A1 was two and three to nine times higher than that of the draining vein and normal brain, respectively, using real-time reverse transcription-polymerase chain reaction. For the first time, here we report the increased expression of ephrin A1 in brain AVM, which may play an important role in the pathogenesis of AVM.

Keywords Angiogenesis · Brain arteriovenous malformation · Ephrin A1 · Gene expression · Vein

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Introduction

Arteriovenous malformation (AVM) of the brain is defined as the direct communication between one or more arteries of the cerebral parenchyma and one or more draining veins without the intervention of a capillary bed [3, 15, 23]. Most brain AVM vessels are composed of large, thin-walled dilated channels displaying fibroblast proliferation and increased cellularity in the wall with focal deposition of elastic tissue layers. They are generally believed to arise when capillaries fail to develop in an area of the brain during early embryogenesis, resulting in abnormal communications between arteries and veins. The growth of blood vessels during embryogenesis and in adult organisms is tightly controlled, and this growth is thought to be mediated by soluble factors [13, 19, 27]. A number of previous studies revealed the abnormal expression of various angiogenesis-related genes or products in brain AVM [7–11, 14, 16, 27].

In order to understand the molecular process of this disease, we attempted to identify differentially expressed

genes in brain AVMs as compared to draining veins, vessels retaining the basic venous architecture [3, 15]. We approached the problem by examining gene-expression profiles. DNA microarray technology nowadays makes it possible to simultaneously analyze the expression patterns of thousands of genes in a given tissue [12, 18, 22]. We here report the identification of ephrin A1, a gene involved in embryogenesis and angiogenesis, which is overexpressed in the nidus of brain AVM.

Materials and methods

Samples and clinical information

After institutional review and informed consent, we obtained brain AVM specimens after resection. One specimen of normal brain was obtained from a temporal lobectomy that was performed for epilepsy. The brain AVM nidus was cut into two pieces for histology and DNA microarray analysis. The nidus was dissected from the adjacent brain tissue in the operating theatre, and a representative portion of nidus tissue was stored at -80°C until analysis. Draining vein specimens were also obtained from the five brain AVM patients. DNA microarray analysis was performed to compare the expression levels of genes between the nidus and draining vein of each patient. The clinical information for patients with brain AVM is shown in Table 1. Three patients had a history of hemorrhage from the brain AVM. None of the patients underwent radiosurgery. Two patients had embolization treatment before surgical resection.

RNA preparation

Frozen sections were placed directly in Trizol reagent (Invitrogen), homogenized for 2 min on ice, and RNA was isolated according to the manufacturer's protocol. RNA was quantified based on absorbance at 260 nm. Total RNA was assessed for quality with an Agilent Lab-on-a-Chip 2100 bioanalyzer. Possible traces of genomic DNA contaminating the RNA preparations were removed by DNase I (Promega) digestion.

Labeling and hybridization

The targets for DNA microarray analysis were prepared according to the manufacturer's instructions. Briefly, cyanine 3- and cyanine 5-labeled complementary RNA (cRNA) was synthesized followed by the production of double-stranded cDNA from 50–500 ng of total RNA with a Human 1A oligoDNA microarray kit (Agilent). After the purification of labeled cRNA, arrays containing 17,086 genes were hybridized with the fragmented cRNA targets at 60°C for 17 h, and then washed and dried with air. We repeated the experiments using the same sample set, but substituting dyes.

Data analysis

Scans were performed using an Agilent DNA microarray scanner (model G2565BA), and the expression value for each gene was calculated by Feature Extraction software. Data were normalized by the LOWESS normalization method. Only the genes deemed present on all slides were further considered. When both cyanine5 and cyanine3 dyes produced signal intensities lower than the cut-off [2.6× standard deviation (SD) of background], we excluded these genes from further analysis. We excluded a sample set when the correlation coefficient for each pair of color swapping was positive. We calculated the mean and SD of $\log_2$ (r1×r2, where r1 and r2 are cy5/cy3 ratios for color swapping) for each pair of sample sets in the entire array, and eliminated pairs of elements whose $\log_2$ (r1×r2) was greater than the SD from the mean. We selected genes whose absolute value for normalized $\log_2$ (cy5/cy3 ratio) was higher than the cut-off value determined in all sample sets examined [1, 25].

Quantitative real time RT-PCR

To evaluate the expression level of the target genes, quantitative RT-PCR was performed using a Sequence Detection System, model 7700 (Perkin Elmer). Quantitative real-time RT-PCR primers were designed using Primer 3. A sense primer and an antisense primer were synthesized using sequences from a database search at NCBI for human ephrin A1 and GAPDH as follows: ephrin A1 sense primer:

Table 1 Clinical features of five patients with brain arteriovenous malformation

Patient	Sex	Age	Grade	Position	Bleeding	Embolization
1	M	53	I	Rt posterior temporal	No	No
2	M	29	V	Lt temporal	Yes	Yes
3	M	35	III	Lt temporal	No	Yes
4	F	33	III	Rt posterior temporal	No	No
5	M	64	II	Lt anterior temporal	Yes	No

TGGAACAGTTCAAATCCCAAG; ephrin A1 antisense primer: CTCATGCTCCACCAGGTACA; GAPDH sense primer: CAGCCTCAAGATCATCAGCA; GAPDH antisense primer: ATCCACAGTCTTCTGGGTGG. Five serial dilutions of each total RNA sample (100, 50, 25, 12.5, and 6.25 ng total RNA) were analyzed for each target gene. Amplification reactions (25 μl) comprised the total RNA samples: 2× SYBR green PCR master mix, 40× MultiScribe reverse transcriptase and RNase inhibitor mix, and 200 nmol/l of each primer. The thermal cycling conditions were as follows: 30 min at 48°C for reverse transcription, 10 min at 95°C for the AmpliTaq Gold activation, 40 cycles of 15 s at 95°C for denaturation, and 1 min at 60°C for annealing and extension. C_T values corresponded to the cycle number at which the fluorescent emission monitored in real time reached the threshold, set at 10 SD above the mean of the baseline emission calculated from cycles 5 to 15. The C_T values decreased linearly with increasing target quantity and could be used as a quantitative measurement of the input target number. Total RNA concentrations from each sample were normalized by the quantity of GAPDH mRNA, and the expression levels of the target genes were evaluated by the ratio of the number of target mRNA to GAPDH mRNA.

Immunohistochemical study

Immunohistochemistry was performed using paraffin-embedded tissue. Heat-induced epitope retrieval was performed in 10 mmol/l citrate buffer. After blocking, the slides were then incubated with a primary antibody. The slides were incubated with a Histofine simple stain MAX-PO (Multi) (Nichirei) for 30 min at room temperature. The slides were washed again and incubated with detecting reagent. The slides were counterstained with hematoxylin. Primary antibodies were polyclonal rabbit anti-human ephrin A1 antibody (Zymed Laboratories). The sensitivity and specificity of primary antibodies were verified using a positive control (prostate) and a negative control (no primary antibody).

Results

One sample set (patient 1, Table 1) was excluded from this study among the five sample sets because the correlation coefficient of color swapping was positive. When we applied an absolute cut-off value for the normalized $\log_2$ (cy5/cy3 ratio) of 0.3, 28 genes were selected; when we applied 0.4, 19 genes were selected. Table 2 shows six upregulated genes in the nidus of brain AVM compared to the draining vein. Table 3 shows 13 genes downregulated in the nidus of brain AVM compared to the draining vein. We examined the signal-intensity ratios of housekeeping genes and genes previously reported to be related to the pathogenesis of brain AVM: ACTB (beta actin); GAPDH (glyceraldehydes-3-phosphate dehydrogenase); VEGF (vascular endothelial growth factor); FLT1 (Fms-related tyrosine kinase 1); ANGPT1 (angiopoietin 1); ANGPT2 (angiopoietin 2); TIE (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains); ENG (endoglin); ACVRL1 (activin receptor-like kinase-1), MMP9 (matrix metalloproteinase 9) and HIF1A (hypoxia-inducible factor 1 alpha subunit). There were no significant differences in gene expression between the nidus of brain AVM and the draining vein (minimum ratio <1, maximum ratio >1) (Table 4). We also examined the signal-intensity ratios of ephrin family genes: EPHA 1–9 (ephrin A 1–9 receptor); EPHB1–6 (ephrin B 1–6 receptor); EFNA 1–6 (ephrin A 1–6); EFNB 1–3 (ephrin B 1–3). Ephrin A6, A9, B4, B5 receptors and ephrin A6 were not detected at all. There were no significant differences in gene expression between the nidus of brain AVM and the draining vein (minimum ratio <1, maximum ratio >1) in other genes of the ephrin family except for ephrin A1. Fig. 1 shows the results of real-time RT-PCR in the draining vein, nidus, and the specimen of normal brain (one sample). The experiment

Table 2 Six genes upregulated in the nidus of brain arteriovenous malformation (AVM) over the draining vein

Probe UID	Symbol	Minimum ratio	Maximum ratio	Gene
8066	SOX8	1.820	3.710	SRY (sex-determining region Y)-box 8
3575	TRIM2	1.622	2.893	Protein with very strong similarity to neural activity-related RING finger (mouse Trim2), member of the B-box zinc-finger family
10794	EFNA1	1.596	2.953	Ephrin-A1
16587	DKFZp761G058	1.554	2.519	Member of the protein-phosphatase 2C family
13012	AQP4	1.519	6.042	Aquaporin 4
7899	APCL	1.429	2.246	Adenomatous polyposis coli-like

We selected genes whose normalized $\log_2$ (signal-intensity ratio, AVM nidus/draining vein) was >0.4 in all cases examined ($\log_2$F 2=1, $\log_2$ 1.32=0.4)

Table 3 Thirteen genes downregulated in the nidus of brain arteirovenous malformation (AVM) over the draining vein

Probe UID	Symbol	Minimum ratio	Maximum ratio	Gene
3484	DACT1	0.265	0.505	Protein of unknown function, has high similarity to uncharacterized mouse Dact1
8938	I_1000105	0.172	0.582	Protein with high similarity to keratin 18 (human KRT18)
17701	KRT18	0.190	0.566	Keratin 18 (cytokeratin 18)
9754	I_931816	0.348	0.559	Protein with high similarity to adipocyte enhancer-binding protein 1 (mouse Aebp1)
10554	WBSCR5	0.442	0.638	Williams-Beuren syndrome chromosome region 5
6480	IGFBP7	0.308	0.647	Insulin-like growth factor binding protein 7
18522	EMILIN-2	0.380	0.659	Extracellular glycoprotein EMILIN-2
7701	RODH	0.419	0.651	Cis-retinol-androgen dehydrogenase 7
1597	FCGR3A	0.460	0.680	Low affinity IIIa receptor for Fc fragment of IgG, neutrophil-specific phosphatidylinositol-linked membrane protein
4594	FLJ10261	0.161	0.702	Member of the DUF590 protein of unknown function family
11152	FSTL1	0.560	0.709	Follistatin-like 1 (follistatin-related protein)
5551	KRT14	0.179	0.726	Keratin 14
8081	I_964005	0.615	0.748	Retired, was a member of the ubiquitin carboxyl-terminal hydrolase family 2

We selected genes whose normalized $\log_2$ (signal-intensity ratio, AVM nidus/draining vein) was <-0.4 in all cases examined ($\log_2$ 0.5=−1, $\log_2$ 0.757=−0.4)

was repeated three times in samples of patients 1, 4, and 5 (see Table 1). The gene expression of ephrin A1 in the nidus was upregulated two and three to nine times higher than that of the draining vein and normal brain specimen. In an immunohistochemical study of eight brain AVMs, expression of ephrin A1 was detected in astrocytes and neurons of the perinidal parenchymal tissue, but not in the surrounding reactive granulomatous tissue nor the normal brain tissue nor in the AVM vessels (Fig. 2).

Discussion

The results of the DNA microarray analysis indicate that not many genes are differentially expressed between the AVM nidus and draining vein. When we applied an absolute cut-off value for the normalized $\log_2$ (cy5/cy3 ratio) of 0.3, only 28 genes were selected; when we applied 0.4, 19 genes were selected. Genes such as SOX8, TRIM2, FENA1 (ephrin A1), and AQP4 related to brain were upregulated. Among the downregulated genes, several genes were related to the extracellular matrix and keratin such as I_1000105, KRT18, IGFBP7, EMILIN-2, and KRT14. We focused on ephrin A1, a gene related to embryogenesis and angiogenesis, among differentially expressed genes detected in this analysis. We confirmed the upregulation of ephrin A1 in the nidus compared to the draining vein and the normal brain tissue using real-time RT-PCR. Our immunohistochemical study showed that ephrin A1 was highly expressed in astrocytes and neurons

Table 4 Signal-intensity ratios of house-keeping genes and genes previously reported to be related to the pathogenesis of brain arteiovenous malformation (AVM)

Probe UID	Symbol	Minimum ratio	Maximum ratio	Gene name
13349	ACTB	0.763	1.806	Beta actin
17973	GAPD	0.959	2.045	Glyceraldehyde-3-phosphate dehydrogenase
1036	VEGF	0.706	1.354	Vascular endothelial growth factor (VEGF)
4744	FLT1	0.755	1.260	Fms related tyrosine kinase 1 (vascular endothelial growth factor-vascular permeability factor receptor)
16472	ANGPT1	0.694	1.203	Angiopoietin 1
2920	ANGPT2	0.432	2.646	Angiopoietin 2
13128	TIE	0.635	1.684	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains
17866	ENG	0.813	1.533	Endoglin
14292	ACVRL1	0.548	1.970	Activin receptor-like kinase-1
11124	MMP9	0.777	1.832	Matrix metalloproteinase 9 (gelatinase B, type IV collagenase)
1103	HIF1A	0.652	2.352	Hypoxia-inducible factor 1 alpha subunit

Ratio indicates AVM nidus/draining vein

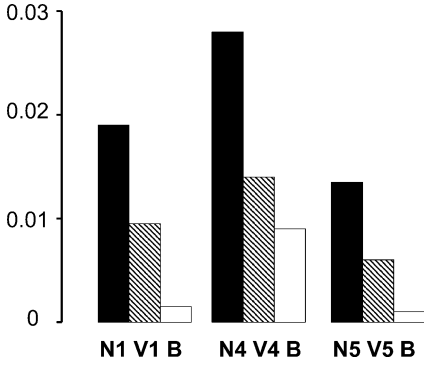

Fig. 1 Gene expressions of ephrin A1 in the draining vein (V), nidus of arteriovenous malformation (N), and normal brain tissue (B). Quantitative real-time RT-PCR was repeated using samples from patients 1, 4, and 5 in Table 1. Expression levels are expressed as relative ratios in C_T values for ephrin A1 to GAPDH

of the perinidal parenchymal tissue, but not in the endothelial or smooth muscle cells of the AVM vessels. Cells in normal brain tissue were not stained by ephrin A1 antibody.

Ephrin A1 was originally identified as a tumor necrosis factor (TNF)-α-inducible gene in human umbilical vein endothelial cells and is expressed in the developing vasculature during embryogenesis [18, 22]. Ephrin A1 induces endothelial cell migration and capillary assembly in vitro and angiogenesis in the corneal pocket assay in vivo [4, 18]. These studies indicate that ephrin A1 is critical for normal blood vessel development and suggest that the molecule may also play a role in angiogenesis for pathological processes in adults. The increasing size of AVMs over time has been documented in the literature [26]. Recurrent AVMs after negative angiograms have also been reported [13]. It is well known that new shunts between arteries and veins grow after embolization of feeding arteries.

We compared our DNA microarray data of angiogenesis-related genes with previously published data. We found no differences in these genes between the AVM nidus and draining vein. The decreased expression of vascular endothelial growth factor receptor (VEGFR), angiopoietin 1 (Ang1) and tyrosin kinase with immunoglobulin and epidermal growth factor homology domains 2 (Tie2), together with an increased expression of Ang2, matrix metalloproteinase (MMP)-9, and tissue inhibitors of metalloproteinases (TIMPs) was reported [7–9]. Hashimoto et al. confirmed a general correlation between many of the previously published findings on abnormal angiogenesis-related protein expression in brain AVM and gene microarray analysis [10]. Sure et al. recently indicated that in AVMs treated with embolization before surgery the expression of HIF1A and VEGF was significantly higher

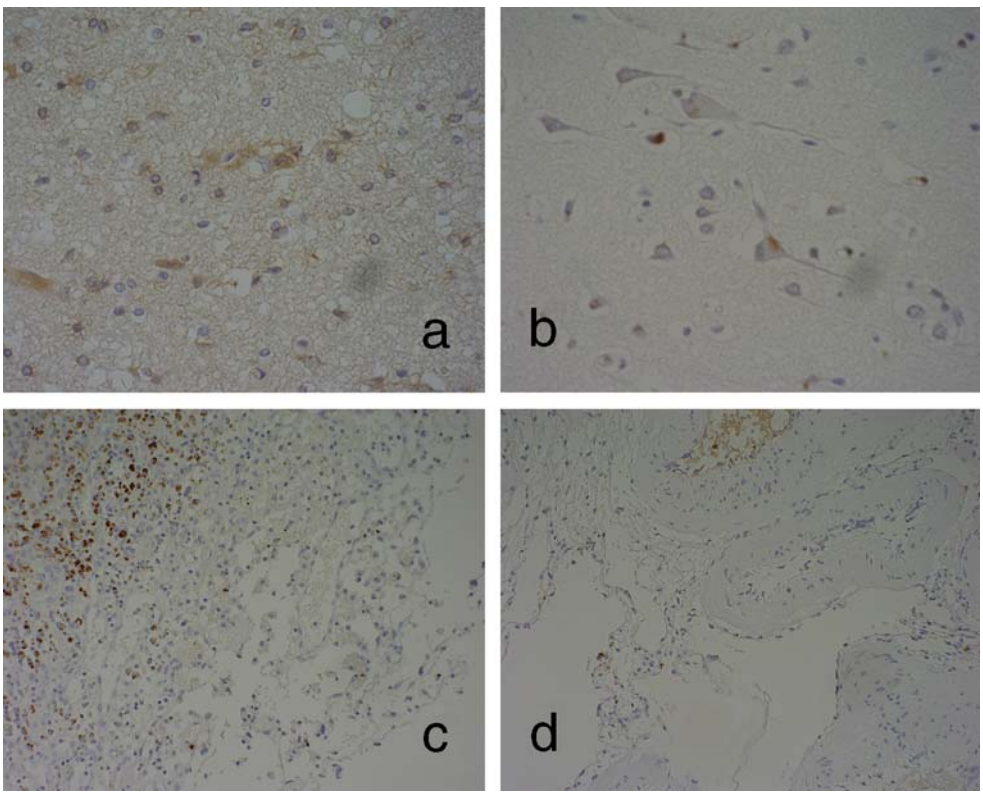

Fig. 2 Localization of ephrin A1 in the nidus of brain AVM: immunohistochemical study. A: Ephrin A1-positive astrocytes in perinidal parenchymal tissue (original magnification 200); B: ephrin A1-positive neurons in the cortex of the perinidal region (×200); C: no ephrin A1-positive cells in the surrounding reactive granulomatous tissue of perinidal parenchyma. Depositions of hemosiderin are seen (×100); D: no ephrin A1-positive cells in AVM nidus (×100)

than in patients who did not have previous endovascular treatment [21]. In our data set there was no significant difference of HIF1A and VEGF between AVM patients with and without embolization before surgery. The difference may be due to the different tissues used for control samples as comparisons to brain AVM. Previous studies all used normal cortex as a control, whereas we selected the draining vein as control, because most of the AVM nidus consists of vessels with a dilated venule-like structure, and the draining vein retains that basic venous architecture. Moreover, our samples are heterogenous in clinical features: the size of the nidus varies, two have a history of bleeding, embolization was performed in two. Gene expressions of the nidus and the vein may be affected. Obviously, it would have been better and more informative if the gene expression levels in the vessel and the brain component of the nidus, the normal vein and the tissue of the normal brain were compared individually.

Among the ephrin family, ephrin A1 was upregulated only in the nidus of brain AVM as compared to the draining vein. The ephrin A1 ligand is expressed predominantly in tumor cells, and ephrin A2 receptors are expressed primarily in a complementary fashion in tumor-associated vasculature [17]. Treatment with soluble EphA2-Fc chimeric receptor results in decreased neovascularization in two different tumor types in vascular window assays, and EphA2-Fc of EphA3-Fc treatment results in decreased tumor vascular density and impaired tumor progression in vivo [2]. Although the pathological state of brain AVM is completely different from tumor neovascularization, a similar approach may elucidate the pathogenesis of brain AVM and help in finding a new treatment for this disease.

Ephrin B2 is expressed in developing arterial endothelial cells, whereas ephrin B4 receptor has a complementary expression and is confined to venous endothelial cells. The ephrin B4 receptor protein and ephrin B2 are therefore considered to define the boundaries between arterial and venous endothelial cells [5, 6]. It was recently reported that endoglin and activin receptor-like kinase-1 (ACVRL1) are necessary for the maintenance of distinct arterial-venous vascular beds and that attenuation of the ACVRL1 signaling pathway is the precipitating event in the etiology of hereditary hemorrhagic telangiectasia, a disease associated with AVM and characterized by the inappropriate fusion of arterioles with venules [20, 24]. We did not detect differences in the expressions of these genes between the AVM nidus and draining vein.

In conclusion, we here report for the first time the increased expression of ephrin A1 in the nidus of brain AVM, which may play important roles in the development and maintenance of AVM.

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Comments

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The authors used DNA microarrays, quantitative PCR and immunohistochemistry to determine if brain AVMs expressed different genes compared to the draining vein of the AVM. Several assumptions are inherent in the design, but the authors have done an excellent series of experiments to document that there is increased expression of ephrin A1 in brain AVMs. The immunohistochemistry suggests it is in the brain tissue around the AVM rather than nidal vessels. The utility of this kind of study is to now provide rationale for additional study of upregulated genes because at this point, one cannot know if the increase is a primary cause of AVM formation or pathogenesis or just a secondary reaction to hemorrhage or the presence of the AVM. However, that is what could be answered in detailed studies in an animal model such as the rat carotid-jugular fistula model [1–4].

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This manuscript describes “Increased expression of ephrin A in brain AVM.” It is a novel and distinguished finding that ephrin A is expressed in neurons or glia in the perinidal parenchymal tissue. This work is one of the continuing studies by Tokyo Women’s Medical University group. Their studies have always been leading the study on gene expression of vascular lesions. This basic study has a chance to open the door to the translational research to the novel strategy for the intractable AVM.

I guess that further study will be appreciated to figure out whether this increased gene expression is the primary pathology or the secondary reaction in the pathological processes. Samples of nidus seem to contain parenchymal tissues, although those of draining veins do not. It would be more informative to study the gene expression profiles of only vascular components in nidus, drainer and normal brain. The detailed analysis of receptor expression for ephrin A in the vascular component would also be very helpful.

I hope that their continuous endeavor to reveal the gene expression in vascular lesion will disclose the unknown mechanism of the formation of AVM and other intracranial vascular lesions. I know that there is a long way in front of us to reach the novel treatment for the difficult AVM. However, without any doubt, I am confident that the authors are leading researchers to develop this difficult task.