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Adjunctive diagnostic tool for histopathological classification of congenital mesoblastic nephroma based in molecular genetic findings

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Abstract

Purpose Congenital mesoblastic nephromas (CMN) are histologically classified into classical, cellular, and mixed subtypes. Most cellular CMNs harbor *ETV6-NTRK3* gene fusions, and classic and mixed CMNs harbor *EGFR* internal tandem duplications (EGFR-ITDs). Classic CMNs are considered benign, whereas recurrent or metastatic diseases occur in the cellular subtypes. Direct identification of mutations is desirable for an accurate diagnosis. However, molecular genetic analyses cannot be performed in a number of histopathology laboratories. This study aimed to investigate a surrogate marker for the accurate histological classification of CMN.

Methods Overall, 11 CMN cases diagnosed at our institute were included in this study. Reverse transcription-polymerase chain reaction was performed for the *NTRK* gene fusion and EGFR-ITDs in all cases. Comprehensive mRNA analysis was performed using the nCounter[®] Gene Expression Assay. Principal component analysis (PCA) was performed based on the gene expression levels. Immunohistochemical evaluation was conducted for the expression of p-Mek1/2, p-Erk1/2, and EGFR.

Results PCA revealed differences in mutation patterns between the EGFR-ITDs and *NTRK* fusion tumor groups. Gene ontology analysis of the highly expressed genes in the EGFR-ITDs tumor group revealed enrichment related to the mitogen-activated protein kinase (MAPK) signaling pathway. p-Mek1/2 and p-Erk1/2 immunoreactivity was significantly increased in the EGFR-ITDs tumor group ($p=0.018$ and $p=0.017$, respectively). EGFR immunoreactivity is not a useful marker for CMN with EGFR-ITD.

Conclusion p-Mek1/2 and p-Erk1/2 immunoreactivity may be useful markers for EGFR-ITDs. Thus, MEK1/2 inhibitors possess the potential to be used as a targeted therapy for CMN with EGFR-ITDs.

Keywords Congenital mesoblastic nephroma · Bioinformatics · Gene expression analysis · Surrogate marker

Introduction

Congenital mesoblastic nephroma (CMN) is the most common renal neoplasm affecting newborns and infants worldwide. Approximately 50–75% of cases occur in the first week of life and are typically discovered within 6 months of age. Therefore, its classification is described as “congenital”. CMN was first described in 1967 as a renal tumor of infancy (Bolande et al. 1967) and is histologically classified into classical, cellular, and mixed subtypes. Histologically, classic CMNs consist of interlacing fascicles of fibroblastic cells with thin tapered nuclei, pink cytoplasm, low mitotic activity and abundant collagen deposition (Mallya et al. 2013). In contrast, cellular CMNs are composed of plump spindles or ovoid cells that histologically mimic malignant

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tumors, with high cellularity, numerous mitoses, apoptotic cells, and necrosis (Charles et al. 1998). Mixed CMN comprise both classical and cellular components. The *ETV6-NTRK3* gene fusion has been identified in cellular CMN (Knezevich et al. 1998; Rubin et al. 1998), establishing both histological and molecular links with infantile fibrosarcoma (IFS).

Recently, most classical and mixed CMN were demonstrated to harbor EGFR-ITDs (Wegert et al. 2018). One study reported IFS with EGFR-ITDs as a driver mutation (van Spronsen et al. 2022). Classic CMNs are considered benign, with recurrent or metastatic disease occurring in the cellular subtypes. The relative risk of recurrence for cellular CMN is higher than that of classic CMN, with a reported incidence of 10% (Gooskens et al. 2017). Thus, it is important to classify CMN into the “*NTRK* fusion tumor group” and “EGFR-ITDs tumor group.” The histological findings of mixed CMN are highly variable and can hinder diagnosis by less experienced pathologists. Although direct identification of mutations using molecular techniques is ideal, many histopathology laboratories in hospitals may not possess easy access to such techniques, particularly for mutations in rare tumors such as CMN. Pan-TRK immunoreactivity is a highly sensitive but not entirely specific diagnostic marker for spindle cell sarcoma with *ETV6-NTRK3* gene fusion (Hung et al. 2018).

This study aimed to investigate a simpler adjunctive tool for accurate histological classification of CMNs. By incorporating more diverse immunohistochemical (IHC) staining patterns and using gene expression analysis, we sought to determine which protein via IHC staining could serve as a surrogate marker in CMN with EGFR-ITDs and *NTRK* rearrangements. Moreover, the traditional thinking is that CMN with EGFR-ITDs is a surgically cured benign disease. However, in rare situations, this is not possible owing to the presence of a single kidney or the other reasons. We also assessed the possibility of another treatment option (neoadjuvant therapy).

Materials and methods

Data source and study patients

Eleven CMN cases from the archive of the Department of Anatomic Pathology at Kyushu University (Fukuoka, Japan) between 1987 and 2023 were included in this study. The patients underwent surgical resection without preoperative treatment. In all cases, the diagnosis was based on light microscopic examination with hematoxylin-eosin staining according to the most recent 5th WHO classification (Argani et al. 2022). The histological diagnoses were reconfirmed

by two pathologists (Y.O. and K.K.) who specialized in the diagnosis of pediatric tumors without knowledge of the clinical data. All patient data were deidentified. Formalin-fixed, paraffin-embedded (FFPE) tissues and fresh frozen tissues with a diagnosis of CMN ($n=11$; cellular CMN=3, classic CMN=5 and mixed CMN=3 cases) were obtained from the archives of our institute. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Kyushu University (approval number: 2021–165).

Reverse transcription-polymerase chain reaction (RT-PCR) for the *NTRK* gene fusion and EGFR-ITD

Total RNA was isolated from FFPE tissues using the CELL-DATA RNastorm FFPE RNA Extraction Kit (Cell Data Sciences, Fremont, CA, USA) and first-strand cDNA was synthesized using the ReverTra Ace qPCR RT kit (Toyobo Biologics Inc., Osaka, Japan) according to the manufacturer's instructions. RT-PCR was performed for the *ETV6-NTRK3* gene fusion as previously described (Yamamoto et al. 2016). As older specimens possessed more fragmented nucleic acids and shorter lengths, two sets of primers yielding amplicon products of different lengths (190 and 100 bp) were designed for EGFR-ITD. The primers flanking the breakpoints for the EGFR-ITD and *ETV6-NTRK3* gene fusions are listed in Supplementary Table 1.

mRNA expression and bioinformatics analysis

To determine differences in gene expression between the *NTRK* fusion tumor group ($n=3$) and the EGFR-ITDs tumor group ($n=8$), comprehensive mRNA expression analysis was performed by NanoString analysis using the nCounter® PanCancer Pathway panel (NanoString Technology, Seattle, USA). The panel analysed the expression of 770 genes representing major cancer pathways, including key driver genes. Gene expression levels in freshly frozen tissues were assessed using 100 ng of total RNA according to the manufacturer's protocol (NanoString Technology). The FFPE tissues were assessed using 300 ng of total RNA. The extracted mRNA samples were enriched for target regions of the nCounter® PanCancer Pathway panel. The raw data were normalized using nSolver™ Analysis Software (version 4.0, NanoString Technology). To gain further insights into the biological functions of the differentially expressed genes (DEGs), we performed gene set enrichment analysis (GSEA). The enrichment score (ES) and normalized enrichment score (NES) were computed using the *NTRK* fusion tumor group as a control. Principal component analysis (PCA) was performed using R version 4.2.3.

Functional enrichment analysis

The DAVID tool (<https://david.ncifcrf.gov/>) was used to calculate Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. *Homo sapiens* was used as the background for the enrichment analysis of all DEGs. Any term with a P -value < 0.05 and gene number ≥ 2 was selected as an enriched term. Fold enrichment and gene expression levels acquired using the NanoString nCounter assay were used to construct the bar graphs. Data were analyzed using the nSolver™ Analysis Software (version 4.0, NanoString Technology) and GeneSpring GX (version 14.8, Agilent Technology) and compared to the controls. Expression values were log2-transformed for statistical analyses.

DEG identification

Differential expression analysis identified the genes with the most statistically significant increase or decrease in expression between the *NTRK* fusion and EGFR-ITDs tumor groups. Genes with an adjusted p value < 0.05 and a log2 fold change ($\log_2FC$) ≥ 1.5 were upregulated in the EGFR-ITDs tumor group. Genes with a $\log_2FC \leq 0.67$ were upregulated in the *NTRK* fusion tumor group.

Immunohistochemistry (IHC) staining

IHC staining was performed using 3- μ m-thick FFPE tissue sections with primary antibodies against Phospho-MEK1/2 (p-Mek1/2) (Ser221) (166F8, rabbit monoclonal, dilution 1:50; Cell Signaling Technology, Danvers, MA), Phospho-p44/42 MAPK (p-Erk1/2) (Thr202/Tyr204) (D13.14.4E, rabbit monoclonal, dilution 1:200; Cell Signaling Technology, Danvers, MA), and EGFR (31G7, mouse monoclonal, dilution 1:50; Invitrogen, Carlsbad, CA). Epitope retrieval was performed in the target retrieval solution (Dako-Japan, Tokyo) for 20 min at 97 °C using a microwave. Each primary antibody was mounted on a tissue section for 1 h at room temperature, and the EnVision FLEX system (Dako) that uses a horseradish peroxidase enzyme-labelled polymer was used to visualize p-Mek1/2 and p-Erk1/2. The EnVision Plus system (Dako) was used for p-MEK1/2 staining. Positive controls were used in parallel. Endothelial cells were strongly positive in all cases and served as internal controls. For antigen retrieval, the slides were pre-digested with protease 1 solution for 32 min and subsequently incubated with pre-diluted monoclonal mouse antibodies against EGFR. The labelled antigens were visualized using 3,3'-diaminobenzidine tetrahydrochloride as the chromogen and the tissue sections were counterstained with hematoxylin. Diffuse

EGFR expression was observed in the membranes of lung tumor cells as a positive control.

The protein expression levels of p-Mek1/2 and p-Erk1/2 were measured using the histochemical score (H-score). The H-score is a histological scoring method used to process IHC staining results. The number of positive cells in each section and the staining intensity were converted into corresponding values to determine cell organization. $H\text{-Score} = \sum (PI \times I) = (\text{percentage of cells of weak intensity} \times 1) + (\text{percentage of cells of moderate intensity} \times 2) + (\text{percentage of cells of strong intensity} \times 3)$. In this formula, PI represents the percentage of positive cells in the section, and I represents the intensity of staining (Liao et al. 2020). EGFR expression was evaluated using proportional expression scores. Immunostaining for EGFR in tumor cells was evaluated for membranous expression as follows: 3+, strong membrane staining $> 10\%$; 2+, moderate membranous staining $> 10\%$; 1+, weak membranous staining $> 10\%$; and 0, negative or $< 10\%$ staining, as previously reported (Williams et al. 2010). We performed IHC staining for EGFR, p-Mek1/2, and p-Erk1/2, and the histological diagnoses were reconfirmed by three pathologists (Y.O., K.K., and H.H.).

Statistical analysis

A p -value of 5% was considered statistically significant unless stated otherwise. Student's t -test was used to compare data between two groups. All statistical analyses were performed using the JMP software (version 16.0; SAS Institute, Cary, NC, USA).

Results

Clinicopathological and genetic findings

The clinicopathological and genetic findings are summarized in Table 1. There were 9 boys and 2 girls. Ten of 11 patients were less than 6 months old at the time of surgery, with a single patient of 29 months. The median age was 1 month. All patients underwent nephrectomy and exhibited favorable outcomes with no evidence of recurrent or metastatic disease, although the follow-up period was limited (24–239 months, median 48 months). The age at surgery was significantly lower in the EGFR-ITDs tumor group than it was in the *NTRK* fusion tumor group ($p = 0.03$). In all three cases of cellular CMNs (3/3; 100%), the *ETV6-NTRK3* gene fusion was detected by RT-PCR. Among the eight cases in the EGFR-ITDs tumor group (three mixed and five classic CMN), seven (7/8; 87.5%) harbored EGFR-ITD as determined by RT-PCR. Case 8 was a classic CMN that did not harbor EGFR-ITD or *ETV6-NTRK3* gene fusions. The

Table 1 Summary of clinicopathological and genetic findings

Case	Pathological type	Sex	Size (cm)	Side	Age at operation	Treatment	Follow up (months)	EGFR-ITD	ETV6-NTRK3 gene fusion
1	Mixed	M	N/A	Rt	11 days	Nephrectomy	N/A	+	-
2	Mixed	M	N/A	Lt	18 days	Nephrectomy	N/A	+	-
3	Mixed	M	8	Rt	1 month	Nephrectomy	239 NED	+	-
4	Cellular	M	N/A	Lt	2 months	Nephrectomy	N/A	-	+
5	Cellular	F	11	Rt	2 years 5 months	Nephrectomy	48 NED	N/A	+
6	Cellular	M	6	Lt	4 months	Nephrectomy	N/A	-	+
7	Classic	M	8	Lt	4 months	Nephrectomy	162 NED	+	N/A
8	Classic	M	9	Lt	27 days	Nephrectomy	N/A	-	-
9	Classic	F	N/A	Rt	16 days	Nephrectomy	N/A	+	-
10	Classic	M	6	Lt	22 days	Nephrectomy	30 NED	+	-
11	Classic	M	3.8	Rt	1 month	Nephrectomy	24 NED	+	-

M indicates male; F, female; Rt, Right; Lt, Left; -, negative; N/A, not available; NED, no evidence of disease

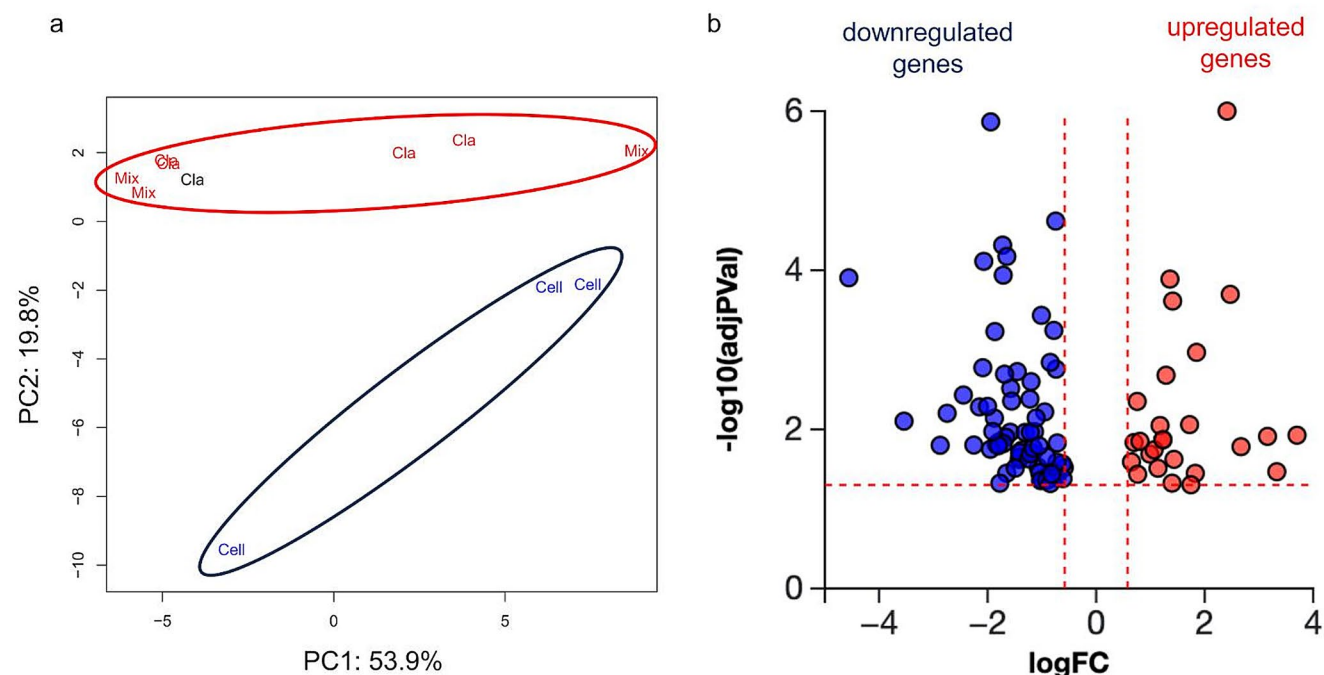


Fig. 1 (a) Principal components analysis (PCA) indicated that PC1 and PC2 exhibited variances of 53.9% and 19.8%, respectively, and that the EGFR-ITDs tumor and *NTRK* fusion tumor groups were separated by PC1 and PC2. In the figure, EGFR-ITDs tumor groups are indicated in red, *NTRK* fusion tumor groups are indicated in blue, and a case of classic CMN lacking EGFR-ITD (case 4) is indicated in black

histological features of the CMNs are presented in Supplementary Fig. 1.

Principal components analysis (PCA)

To confirm the relevance of the two groups, we performed PCA using gene expression levels acquired from the nCounter® Gene Expression Assay (Fig. 1a). We analyzed

(b) The up-regulated genes (positive log fold change) are indicated in red, while the down-regulated genes are indicated in blue color. All 95 DEGs are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis). The dotted lines represent the thresholds used to select the DEGs, with 0.58 for expression change and 0.05 for significance

the PCA after removing 70 genes with low expression from the 770 genes associated with major cancer pathways and observed a separation between the EGFR-ITDs tumor group and the *NTRK* fusion tumor group. A case (case 8) lacking EGFR-ITD (marked as “Cla” in black) was located in the EGFR-ITDs tumor group.

Table 2 GO analysis of the upregulated genes in the EGFR-ITDs tumor group

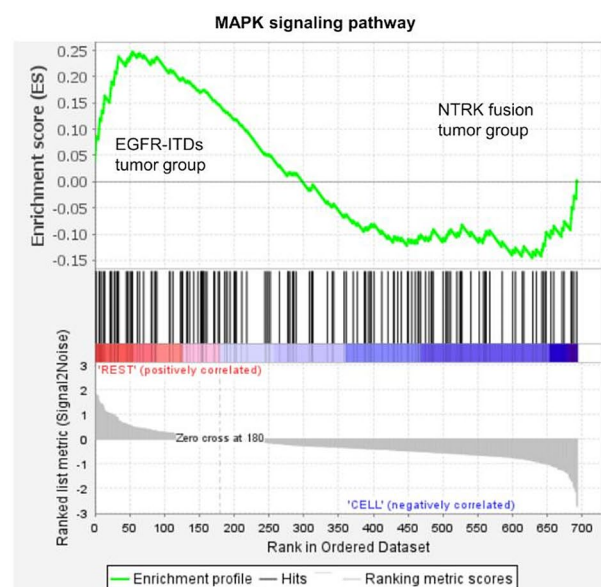
Functional and pathway enrichment analysis of upregulated genes in EGFR-ITDs tumor group					
Category	Term	Count	%	P-value	FDR
KEGG_PATHWAY	hsa04010:MAPK signalling pathway	11	42.3	4.60E-09	5.33E-07
KEGG_PATHWAY	hsa04510:Focal adhesion	9	34.6	6.10E-08	3.28E-06
KEGG_PATHWAY	hsa05200:Pathways in cancer	12	46.2	8.40E-08	3.28E-06
KEGG_PATHWAY	hsa04151:PI3K-Akt signalling pathway	10	38.5	3.70E-07	1.08E-05
KEGG_PATHWAY	hsa05223:Non-small cell lung cancer	5	19.2	4.90E-05	1.04E-03

Count: Number of enriched genes in each term

Top five terms were selected per the *P* value

Bioinformatics analysis

In the current analysis, 95 DEGs were identified from the 770 genes with measured expression levels. All the 95 significant DEGs are presented as volcano plots (Fig. 1b). A Log₂ (fold change) (log₂FC) > 0.58 and a corrected *p* value < 0.05 were used as the cutoff criteria for DEGs. Among the 95 DEGs, 26 were significantly upregulated in the EGFR-ITDs tumor group (Supplementary Table 2). The upregulated DEGs are plotted in the upper right panel of Fig. 1b.

**Fig. 2** In the figure, Gene Set Enrichment Analysis (GSEA) of the MAPK signaling pathway genes enriched in the EGFR-ITDs tumor group compared to that in the *NTRK* fusion tumor group as control

To further understand the function and mechanism of the identified DEGs, functional and pathway enrichment analyses, including GO and KEGG, were performed using DAVID. GO term enrichment analysis demonstrated that the upregulated genes in the EGFR-ITDs tumor group were significantly enriched in the MAPK signaling pathway (Table 2). Moreover, the results of pathway enrichment analysis using GSEA indicated significant enrichment in the MAPK signaling pathway (ES=0.25, NES=1.34) (Fig. 2). Therefore, we validated the expression levels of shortlisted genes associated with the MAPK signaling pathway. The expression levels of *MAPK3*, *FGF7*, and *JUN* were significantly higher in classic than they were in cellular CMNs (Supplementary Fig. 2).

Immunohistochemical results

p-Mek1/2 and p-Erk1/2

Representative IHC staining for p-Mek1/2 and p-Erk1/2 is presented in Fig. 3. In the EGFR-ITDs tumor group cells, p-Mek1/2 expression was diffusely positive in the cytoplasm, and p-Erk1/2 expression was diffusely positive in the nuclei. However, tumor cells in the *NTRK* fusion tumor group were negative for p-Mek1/2 and p-Erk1/2 expression. The H-score of p-Mek1/2 and p-Erk1/2 were significantly higher in the EGFR-ITDs tumor group than in the *NTRK* fusion tumor group (*p*=0.018 and *p*=0.017, respectively) (Fig. 4 and Supplementary Table 3).

TERM	ES	NES	p-value
KEGG_ECM_RECEPTOR_INTERACTION	0.65	2.62	0.000
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	0.60	2.33	0.000
KEGG_DILATED_CARDIOMYOPATHY	0.57	2.25	0.000
KEGG_FOCAL_ADHESION	0.37	1.91	0.000
KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC	0.48	1.78	0.019
KEGG_TGF_BETA_SIGNALING_PATHWAY	0.36	1.67	0.000
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	0.31	1.54	0.016
KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION	0.35	1.37	0.074
KEGG_MAPK_SIGNALING_PATHWAY	0.25	1.34	0.019

is presented. The enriched terms of pathways are listed. The MAPK signaling pathway (ES=0.25, NES=1.34) is indicated in red

Fig. 3 Classic CMN of a 1-month-old boy (case 11), where p-Mek1/2 expression was primarily detected in the cytoplasm of the tumor cells (the figure to the left). p-Erk1/2 expression was detected with diffuse cytoplasmic and strong nuclear expression in the tumor cells (the figure to the right)

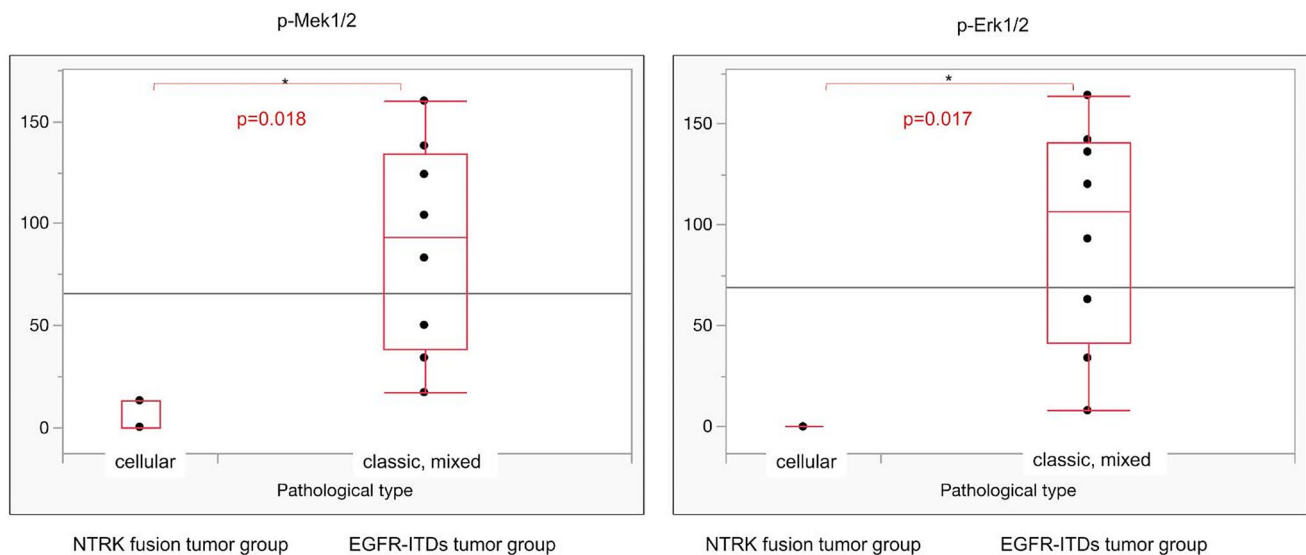
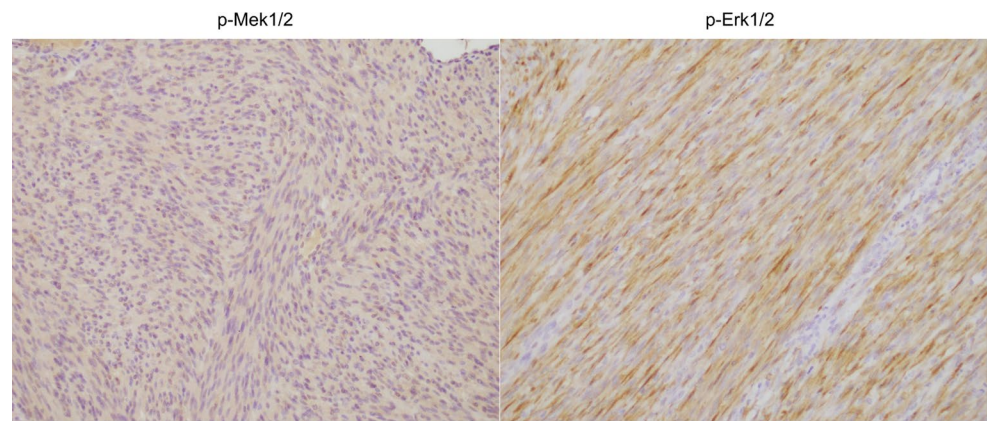


Fig. 4 The distribution of the H-scores is presented in the box plot. The horizontal bar indicates the median value. The expression of p-Erk1/2 and p-Mek1/2 were significantly increased in the EGFR-ITDs tumor group (0 vs. 95 ± 55.5 ; $p=0.017$ and 4.3 ± 7.5 vs. 88.7 ± 51.6 ; $p=0.018$,

respectively). Immunostaining of CMN clinical samples demonstrated that the EGFR-ITDs tumor group expressed higher levels of p-Erk1/2 and p-Mek1/2 than did the *NTRK* fusion tumor group. * ($p<0.05$) indicates statistically significant difference compared to control

EGFR

To determine if EGFR protein expression could be used as a surrogate marker to identify EGFR-ITD cases, we performed EGFR IHC staining on all 11 CMNs. Nine (81.8%) of 11 CMNs exhibited membranous EGFR expression. Most of the positively stained cases (6/9) exhibited EGFR-ITDs. However, two of the three patients lacking EGFR-ITD also exhibited positive staining. Of the eight positively stained cases, there were six cases of weak membrane staining (1+), two cases of intermediate membrane staining (2+), and no cases of strong complete membrane staining (3+) (Supplementary Fig. 3a–c). Neither the gene expression level nor the proportional expression score of EGFR correlated with the presence of EGFR-ITD (Supplementary Fig. 3d).

Discussion

A novel finding of our study was that the gene expression analysis of highly expressed genes in the EGFR-ITDs tumor group revealed enrichment related to the MAPK/ERK pathway. This study evaluated the IHC expression of p-Mek1/2, p-Erk1/2, and EGFR in 11 CMN samples and correlated them with the clinicopathological characteristics. IHC analysis demonstrated that the levels of p-Mek1/2 and p-Erk1/2 immunoreactivity increased in the EGFR-ITDs tumor group (classic and mixed CMN) that harbors EGFR-ITDs. These findings suggested that p-Mek1/2 and p-Erk1/2 immunoreactivity may be useful surrogate markers for EGFR-ITDs.

To date, few studies have assessed the expression of p-Mek1/2, p-Erk1/2, and EGFR in CMN. For rare renal diseases such as CMNs, new diagnostic tools can be developed by incorporating more diverse IHC staining patterns. Although

pan-TRK IHC staining has been recommended as a first-line screening test for soft tissue tumors with suspected *NTRK* rearrangements, a recent review suggested that the specificity of pan-TRK IHC staining may be insufficient. (Hung et al. 2018; Zhao et al. 2020) In this study, the H-score of p-Mek1/2 and p-Erk1/2 were significantly higher in the EGFR-ITDs tumor group such as classical and mixed cellularity CMN, respectively. Thus, p-Mek1/2 and p-Erk1/2 IHC staining may be useful for identifying EGFR-ITDs. In contrast, we observed no correlation among *EGFR* expression, EGFR staining, and EGFR-ITDs. These results indicate that EGFR immunoreactivity is not useful as a marker for CMN with EGFR-ITD or for distinguishing CMN from other tumor types, and this is in agreement with previous studies (Zhao et al. 2020).

Patients with Wilms' tumor may require chemotherapy and radiotherapy depending on the disease stage. However, CMN, the most common renal tumor in infancy, is typically treated with surgical resection, which is the standard treatment for CMN. However, in rare cases this may not be possible due to the presence of a single kidney or if symptoms indicate a high risk of surgical or perioperative complications. Neoadjuvant chemotherapy is only recommended for patients with recurrent disease or if the lesion is inoperable. In such situations, kinase inhibitor therapy is useful for targeting activated kinases and preventing overactive signaling. (Schram et al. 2017) Targeted therapy such as larotrectinib (LOXO-101) and entrectinib in the presence of the *NTRK* fusion is an exciting new possibility for children with cellular CMN with recurrence and metastasis (Pavlick et al. 2017). The successful use of larotrectinib in IFS harboring *ETV6-NTRK3* fusion has been previously reported (Laetsch et al. 2018). However, few studies have reported targeted therapies for EGFR-ITD tumors. Interestingly, using mRNA expression and bioinformatic analyses, we determined that the MAPK signaling pathway was more frequently activated in the EGFR-ITDs tumor group. Furthermore, the expression of genes associated with the MAPK signaling pathway such as *MAPK3*, *FGF7* and *JUN* was significantly higher in classic CMNs than it was in cellular CMNs. Thus, MEK1/2 inhibitors have the potential to be used as targeted therapies for CMN patients with EGFR-ITDs.

The MAPK/ERK signaling pathway regulates several processes involved in cell proliferation and survival. *KRAS* mutations result in constitutive activation of this pathway, and selumetinib (AZD6244, ARRY-142886) is a potent and selective inhibitor of MEK1/2 (Wang et al. 2021). Targeting MEK has become an important strategy for cancer therapy, and four MEK inhibitors have been approved by the FDA to date, including Trametinib (approved in 2013 for patients with melanoma) (Banks et al. 2017), Cobimetinib (2015, *BRAF*-mutant advanced melanoma) (Gauci et al. 2017), Binimetinib (2018, unresectable or metastatic melanoma with a *BRAFV600E* or *-BRAFV600K* mutation) (Shirley 2018), and Selumetinib

(2020, neurofibromatosis type 1, data from www.accessdata.fda.gov). In a structure-function analysis of oncogenic EGFR-ITD, Du et al. reported that, similar to the molecular biological background of classic CMN, EGFR-ITD mutants exhibited higher phosphorylation and *EGF*-independent activation states, leading to aberrant EGFR signaling (Du et al. 2021). Another study demonstrated that the EGFR-ITD can be therapeutically targeted with EGFR TKIs such as Erlotinib, afatinib, and AZD9291 (Gallant et al. 2015).

EGFR activation may occur via alternative mechanisms in patients with CMN lacking EGFR-ITD (Lei et al. 2020). In our study, PCA of next-generation genomic sequencing data indicated differences in mutation patterns between the EGFR-ITDs and *NTRK* fusion tumor groups. Notably, case 8 in our series (a classic CMN lacking EGFR-ITD) was plotted in the EGFR-ITDs tumor group. The presence of a fusion transcript in the classical area of mixed CMN has not been previously demonstrated. Therefore, the molecular features of mixed CMN are identical to those of classic CMN, suggesting that the cellular component of mixed CMN may be a cytological variant of the classic component.

This study has several limitations. First, it is a very small group of cases. Second, in general CMN is a relatively straightforward diagnosis on hematoxylin and eosin sections, for which molecular analysis is not necessary in many instances. Only in selected cases, for instance when only a biopsy is received of a cellular tumor, in a child over 6 months, or in the case of an inexperienced observer, ancillary techniques become important. Third, we have not studied other pediatric renal tumors that may enter the differential diagnosis, and we have not done parallel pan-TRK IHC. As follow-up was limited in our cases, it was not possible to indicate the prognostic value of the presence of an *NTRK* fusion tumor group compared to that of an EGFR-ITDs tumor group in CMN. As CMNs are rare, it should be noted that CMN cases with *NTRK* fusion exhibit a better prognosis than do fusion-negative cases. In conclusion, our findings indicate that p-Mek1/2 and p-Erk1/2 may serve as highly sensitive diagnostic markers for classic and mixed CMN. Future studies are required to confirm our findings regarding the morphology and prognosis of EGFR-ITD-related lesions as well as the possible role of a MEK1/2 inhibitor against EGFR-ITDs.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00432-025-06116-x>.

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Author contributions All the authors contributed to the conception and design of this study. Hiroshi Hamada, Kenichi Kohashi, and Takeshi

Iwasaki performed the material preparation, data collection, and analysis. Hiroshi Hamada wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All the authors have read and approved the final version of this manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval The present study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Kyushu University (approval codes: 2021–165).

Informed consent Informed consent was obtained from all participants included in this study.

Competing interests The authors declare no competing interests.

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