NOTCH1 pathway activating mutations and clonal evolution in pediatric T-cell acute lymphoblastic leukemia

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Molecular mechanisms involved in the relapse of T-cell acute lymphoblastic leukemia (T-ALL) are not fully understood, although activating NOTCH1 signaling due to NOTCH1/FBXW7 alterations is a major oncogenic driver. To unravel the relevance of NOTCH1/FBXW7 mutations associated with relapse, we performed whole-exome sequencing in 30 pediatric T-ALL cases, among which 11 diagnosis-relapse paired cases were further investigated to track the clonal evolution of relapse using ampli-con-based deep sequencing. NOTCH1/FBXW7 alterations were detected in 73.3% (diagnosis) and 72.7% (relapse) of cases. Single nucleotide variations in the heterodimerization domain were the most frequent (40.0%) at diagnosis, whereas proline, glutamic acid, serine, threonine-rich (PEST) domain alterations were the most frequent at relapse (54.5%). Comparison between non-relapsed and relapsed cases at diagnosis showed a predominance of PEST alterations in relapsed cases (P = .045), although we failed to validate this in the TARGET cohort. Based on the clonal analysis of diagnosis-relapse samples, we identified NOTCH1 “switching” characterized by different NOTCH1 mutations in a major clone between diagnosis and relapse samples in 2 out of 11 diagnosis-relapse paired cases analyzed. We found another NOTCH1 “switching” case in a previously reported Berlin-Frankfurt-Münster cohort (n = 13), indicating NOTCH1 importance in both the development and progression of T-ALL.
Despite the limitations of having a small sample size and a non-minimal residual disease-based protocol, our results suggest that the presence of NOTCH1 mutations might contribute to the disease relapse of T-ALL.

**KEYWORDS**

NOTCH1, pediatric leukemia, relapse, T-cell acute lymphoblastic leukemia, whole-exome sequencing

1 | INTRODUCTION

Although pediatric cases of T-cell acute lymphoblastic leukemia (T-ALL) have a cure rate exceeding 80% because of intensified chemotherapies and appropriate prognostic classifications, the outcome of T-ALL patients with primary resistant or relapsed leukemia remains extremely poor. Recent insights into the biology of the disease have uncovered the genomic landscape of T-ALL at diagnosis and defined T-ALL subgroups. However, the molecular basis of refractory/relapsed T-ALL is largely unknown, except for the involvement of NT5C2 mutations in relapsed T-ALL. In addition, the underlying clonal evolution leading to relapse and treatment resistance has been poorly studied in pediatric T-ALL. In contrast, NOTCH1 and/or FBXW7 alterations, leading to constitutive activation of NOTCH1 signaling, are among the most common changes detected in T-ALL patients. It is well known that alterations in the negative regulatory region (NRR), such as single nucleotide variations in the heterodimerization (HD) domain (HD-SNV) and small in-frame insertions or deletions in the HD domain (Indel), lead to constitutive activation of NOTCH1 without ligand binding. NOTCH1 transcriptional activation is terminated by the proteasomal degradation of the NOTCH1 intracellular domain (NICD), which is induced by the proline, glutamic acid, serine, threonine–rich (PEST) domain recognition of the FBXW7-SCF ubiquitin ligase complex. Alterations in the PEST domain of NOTCH1 and FBXW7 result in impaired degradation (ID) of NICD, leading to aberrantly prolonged NOTCH1 signaling. Thus, there are 2 patterns of activation of NOTCH1 signaling: ligand–independent activation (LIA) of NOTCH1 and ID of NOTCH1.

Although activated NOTCH1 signaling constitutes the most predominant oncogenic event involved in the pathogenesis of T-ALL, it is widely reported that T-ALL patients with NOTCH1 mutations have a favorable early therapeutic response or outcome. However, some groups have reported no effect of NOTCH1 mutations in the outcome of T-ALL. Thus, the prognostic relevance of NOTCH1 and/or FBXW7 alterations is still controversial and may be protocol–specific. Moreover, the role of NOTCH1 and/or FBXW7 mutations in T-ALL relapse is unclear. Despite recent advances in genome–wide analyses of diagnostic T-ALL, little is known about the involvement of NOTCH1 in the relapse and progression of T-ALL. To investigate relapse–related genes in the progression of T-ALL from diagnosis to relapse, especially the impact of NOTCH1 and/or FBXW7 alterations, we performed genetic analysis of 30 pediatric T-ALL cases using whole-exome sequencing (WXS) and amplicon–based deep sequencing. Among these 30 cases, 12 cases were relapsed, and 11 diagnosis-relapse paired cases available were further investigated to track the clonal evolution of the relapses.

2 | MATERIALS AND METHODS

2.1 | Patients and materials

Thirty pediatric T-ALL patients were enrolled in this study (Table S1); results for 24 cases were reported previously. Analyzed samples were mainly offered from the Tokyo Children’s Cancer Study Group (TCCSG) and the Japan Association of Childhood Leukemia Study (JACLS). All patients received Berlin- Frankfurt-Münster (BFM)–based chemotherapy. No minimal residual disease (MRD)–based risk stratification was performed. Written, informed consent was attained according to protocols approved by the Human Genome, Gene Analysis Research Ethics Committee of the University of Tokyo and other participating institutes. Peripheral blood, bone marrow blood and lymph node samples were collected from T-ALL patients. Among 12 relapsed cases, relapse samples were available in 11 cases.

2.2 | Whole-exome sequencing

Whole–exome sequencing of diagnosis/relapse tumor and matched normal specimens was performed as previously described. Whole–exome capture was accomplished using SureSelect Human All Exon Kit V3 or V5 (Agilent Technology, Wilmington, DE) and was subjected to sequencing using HiSeq 2000 (Illumina, San Diego, CA) according to the manufacturer’s protocol. Raw sequence data were processed using our in–house pipelines (Genomon 2.3.0, https://github.com/Genomon-Project/GenomonPipeline). Sequence reads with a mapping quality score <25, base quality score <30, or 5 or more mismatched bases were excluded. Relevant somatic mutations were filtered by excluding variants: (i) with incomplete open reading frame information; (ii) listed in the 1000 Genomes Project (May 2011 release), NCBI SNP database (dbSNP) build 131, National Heart, Lung, and Blood
designed (Table S2). Target sequences were amplified using primers given the predetermined bait design, primers were specifically described previously.27 PCR products were ligated and fragmented for deep sequencing as variational Bayesian beta mixture model in SciClone 31 assessed using Student’s 2–tailed t test, and resulting values were considered statistically significant at \( P < .05 \).

3.1 | Mutation detection at diagnosis and relapse of T-cell acute lymphoblastic leukemia using whole-exome sequencing

We performed WXS on 30 cases of matched T-ALL and normal (samples in complete remission phase) pairs, including 11 trios comprising samples at relapse, diagnosis, and normal (Table S1). One T-ALL case (TALL023) developed Langerhans cell histiocytosis (LCH) after achieving complete remission. In this case, the LCH cells are not only a derivative of the same ancestral clone but should be a branched clone from the one that already gave rise to T-ALL, because the LCH cells have the same TCR rearrangements (Figure S1D,E).35,36 WXS was performed at an average coverage of 107.8× (range: 66.2-201.9) (Figure S2). All predicted SNVs were validated by amplicon–based deep sequencing (an average coverage of 7942×). Across the coding regions of 30 cases (total 41 samples: 30 samples at diagnosis and 11 samples at relapse), we detected 777 somatic mutations and structural variants in 515 genes (Tables S3-S4). Each T-ALL sample at diagnosis harbored a mean of 16.9 somatic mutations (range: 3-29), of which the mean number of mutations without synonymous SNV was 14.4 per sample (range: 2-24). The mutation rate of relapse samples was significantly higher than that of samples at diagnosis, with a mean of 24.6 mutations per sample (range: 9-40, \( P = .017 \); Figure S3) and a mean of 20.5 mutations without synonymous SNV per sample (range: 9-31; \( P = .014 \); Figure 1A). No difference was observed between non–relapsed and relapsed cases in the mutation rate of diagnostic T-ALL samples (Figure 1A and Figure S3). Because of the small sample number of cases analyzed, we could not find different features of immunophenotype and molecular subgroups between non–relapse and relapse samples (Table 1 and Figure S4). These subgroups were defined in our previous study6 based on expression data of whole–transcriptome sequencing. Consistent with previous reports, mutations in NOTCH1 (66.7%), FBXW7 (20.0%), DNM2 (20.0%) and PHF6 (20.0%) were common in T-ALL samples at diagnosis (Figure 1B and Figure S5A). Among these, NOTCH1 (72.7%) mutations were also frequently observed in relapsed T-ALL, but FBXW7, DNM2 and PHF6 mutations were less frequent. As previously reported, NT5C2 mutations (27.3%) were predominantly detected in relapse samples (Figure 1C and Figure S5B). An overrepresentation of C>T and C>A transitions was observed in samples at both diagnosis and relapse (Figure S6).

3.2 | Germline mutations in predisposition genes detected in whole-exome sequencing

We identified 3 missense germline variants, FAP (NM_004460: K533X), IDH1 (NM_005896: F355V) and RET (NM_020630: M848V), in 2 specimens (Table S5). FAP, encoding a tumor suppressor involved in cell proliferation and survival, was truncated at exon 18. The IDH1 variant was reported as one of the cancer predisposition tumor suppressor genes.27 The detected IDH1 variant was predicted as a high
risk factor for developing cancers according to the mutation assessor (http://mutationassessor.org/v1/), although no germline and somatic mutations have been reported at the same position. The mutation in RET was located in its kinase domain, which is consistent with previous reports.\(^{37}\) Importantly, neither family history of cancer nor underlying disease was observed in these cases.

### 3.3 | Types of NOTCH1 and FBXW7 mutations observed in T-cell acute lymphoblastic leukemia

We detected mutations leading to NOTCH1 signaling activation (NOTCH1 and/or FBXW7 alterations) in 22 out of 30 (73.3%) and 8 out of 11 (72.7%) samples at diagnosis and relapse, respectively (Figure 2A and Table S3-S4). Both LIA and ID mutation types were detected in 11 out of 30 (36.7%) and 3 out of 11 (27.3%) samples at diagnosis and relapse, respectively (Figure 2A, B and Figure S7-S8). These mutations were mutually exclusive within each LIA and ID pattern, except for 1 sample that harbored mutations with low allele frequencies less than 15% (Figure 2A). In our cohort, HD-SNVs were the most frequent (40.0%; 12 out of 30 samples), followed by PEST alterations (33.3%; 10 out of 30 samples). In-frame internal duplication in exon 28, known as juxta-membrane expansion (Dup), intragenic NOTCH1 deletion (Del-N) and SNV at 3′UTR (UTR) were each detected in 1 sample (Table 2). The frequency of PEST alterations in relapsed cases of diagnosis samples (7 out of 12; 58.3%) was significantly higher than that in non-relapsed cases (3 out of 18; 16.7%) (\(P = .045\); Figure 2C and Table 2). We also analyzed the WXS data of 200 T-ALL cases generated by Therapeutically Applicable Research to Generate Effective Treatment (TARGET)\(^{7}\); however, no predominance of PEST alterations was observed in relapsed cases (Table S6).

### 3.4 | Clonal analysis of samples at diagnosis and relapse identified mutational "switching" of NOTCH1

Based on the results of amplicon-based deep sequencing of all mutations detected by WXS, we evaluated the clonal evolution of T-ALL relapses using accurate VAFs of each detected mutation. The clonal analysis of diagnosis and relapse samples identified 3 different types of clonal evolution at relapse in T-ALL samples: acquired new mutations (VAF >.01) at relapse which were not detected or very low level (VAF <.01) at diagnosis without losing any mutations (VAF
TABLE 1 Clinical characteristics of relapse and non-relapse cases at diagnosis

| Characteristics          | Non-relapse cases (N = 18) | Relapse cases (N = 12) | Fisher’s exact test P-value |
|--------------------------|---------------------------|-----------------------|-----------------------------|
| Sex                      |                           |                       |                             |
| Male                     | 13 (72.2)                 | 10 (90.9)             |                             |
| Female                   | 5 (27.8)                  | 1 (9.1)               | n.s.                        |
| Not available            | 0                         | 1                     |                             |
| Age at diagnosis         |                           |                       |                             |
| <10 y                    | 11 (61.1)                 | 5 (41.7)              |                             |
| ≥10 y                    | 7 (38.9)                  | 7 (58.3)              |                             |
| Not available            | 0                         | 0                     |                             |
| WBC counts at diagnosis  |                           |                       |                             |
| <10 000/μL               | 12 (66.7)                 | 4 (33.3)              |                             |
| ≥10 000/μL               | 6 (33.3)                  | 8 (66.7)              |                             |
| Not available            | 0                         | 0                     |                             |
| Prednisolone response    |                           |                       |                             |
| Good (day 8 WBC<1000/μL) | 9 (52.9)                  | 8 (66.7)              |                             |
| Poor (day 8 WBC≥1000/μL) | 8 (47.1)                  | 2 (20.0)              |                             |
| Not available            | 1                         | 2                     |                             |
| CNS involvement          |                           |                       |                             |
| CNS-1, CNS-2             | 17 (94.4)                 | 10 (90.9)             |                             |
| CNS-3                    | 1 (5.6)                   | 1 (9.1)               | n.s.                        |
| Not available            | 0                         | 1                     |                             |
| Mediastinal mass         |                           |                       |                             |
| Yes                      | 7 (38.9)                  | 7 (58.3)              |                             |
| No                       | 11 (61.1)                 | 5 (41.7)              |                             |
| Not available            | 0                         | 0                     |                             |
| Previously analyzed expression cluster^4 | |                       |                             |
| TAL1-RA                  | 5 (33.3)                  | 4 (44.4)              |                             |
| TAL1-RB                  | 2 (13.3)                  | 0 (0)                 |                             |
| TLX                      | 3 (20.0)                  | 1 (11.1)              |                             |
| ETP                      | 4 (26.7)                  | 2 (22.2)              |                             |
| SPI1-fusion              | 1 (6.7)                   | 2 (22.2)              |                             |
| Follow-up duration days (median) | 507-5427 (3069) | 209-3211 (750) |                             |

CNS, central nervous system; ETP, early T-cell precursor; WBC, white blood cell.

^4These 5 clusters were identified using whole-transcriptome sequencing based on expression profiles in our previous study. ETP, immature cluster showing similar expression profiles to early T-cell precursor; SPI1-fusion; SPI1-highly expressed cluster with SPI1 fusion; TAL1-RA, TAL1-highly expressed cluster with low gene mutation rate; TAL1-RB, TAL1-highly expressed cluster with high gene mutation rate; TLX, TLX1/TLX3-highly expressed cluster.

>0.01 (type 1; Figure 3A), acquired new mutations (VAF >0.1) and lost some mutations (VAF <0.1) at relapse (type 2; Figure 3B); and no mutations were acquired (VAF >0.1) or lost (VAF <.01) at relapse (type 3; Figure 3C).

Type 1 clonal evolution at relapse was observed in 3 samples (TALL001, TALL015 and TALL026). These samples acquired additional mutations later in the process of leukemogenesis at relapse. All mutations detected at diagnosis were also detected in relapse samples (Figure 3A-D). NOTCH1 and/or FBXW7 alterations were detected in the common major clone (VAF >.15, both diagnosis and relapse) in TALL001 and TALL015, and none of these 3 type 1 cases acquired additional NOTCH1 and/or FBXW7 alterations at relapse. Unlike the previous report, all cases of type 1 clonal evolution relapsed after 12 months of diagnosis (Figure S9A).

In type 2 clonal evolution, several mutations detected in the major clone at diagnosis were lost and additional new mutations were acquired at relapse (Figure 3E-H). Type 2 clonal evolution was observed in 7 cases (TALL002, TALL003, TALL005, TALL017, TALL023, TALL029 and TALL031), of which PEST alterations were identified in 6 cases at relapse. Three cases also acquired HD-SNVs and GATA3 mutations at the same time. In TALL031, PEST alteration was detected in the common major clone both at diagnosis and relapse. The other major clone (Clone 2) at diagnosis disappeared and the new major clone (Clone 3) with the acquired NOTCH1 mutation (HD-SNV) expanded at relapse (Figure 3E). In contrast, in TALL029, both the HD-SNV and PEST alterations were detected at diagnosis; of these, the HD-SNV disappeared and only the PEST alteration was identified with other acquired mutations at relapse.

It should be noted that we identified NOTCH1 "switching" in 2 cases (TALL017 and TALL023) with type 2 clonal evolution, showing different NOTCH1 mutations at diagnosis and relapse. In these cases, the dominant clone at diagnosis with NOTCH1 mutations had disappeared at relapse with an expansion of a new dominant clone with a different NOTCH1 mutation derived from the same ancestor clone (Figure 4 and Figure S1). For example, TALL017 harbored 3 NOTCH1 mutations at diagnosis, PEST alteration in Clone 1 (common major clone, VAF >.15 both at diagnosis and at relapse), and 2 independent HD-SNVs in Clone 2 (VAF >.15 at diagnosis) and Clone 3 (VAF <.15 at diagnosis), respectively (Figure 4A). The treatment of T-ALL at diagnosis might eradicate 1 of the HD-SNVs present in Clone 2. The other HD-SNV in Clone 3 expanded as a "switched" major clone (VAF >.15) at relapse (Figure 4A-D). In addition to NOTCH1 expansion, NT5C2 mutation was also acquired in this "switched" clone, and seemed to have a selective advantage for relapse (Figure 4D). Intriguingly, TALL023 was the other case of NOTCH1 "switching" that developed LCH from a common clone in diagnostic T-ALL (Figure 4E-H and Figure S1). In the T-ALL sample, 2 NOTCH1 mutations (both with ID pattern) were identified in the UTR (Clone 2, VAF = .19) and the other in the PEST domain (Clone 4, VAF = .07). In the LCH sample, only the PEST alteration was observed, suggesting that the mutation in the UTR disappeared after the initial T-ALL treatment.
Moreover, the clone with PEST alteration (Clone 4) expanded as a "switched" major clone (VAF >.15) at LCH from a minor subclone (VAF <.15) in the T- ALL sample at diagnosis (Figure 4E-H). In this case, the acquisition and expansion of \( \text{BRAF} \) alteration, important for the pathogenesis of LCH, were observed at LCH in addition to \( \text{NOTCH1} \) "switching." By contrast, the \( \text{GATA3} \) mutation involved in T- ALL pathogenesis was eradicated. Using multiplex PCR for the detection of TCR rearrangement between diagnosis and relapse samples of these "switching" cases, we confirmed that diagnosis-relapse paired samples had the same TCR rearrangement pattern (Figure S1). Intriguingly, we detected multiple waves in TALL017 that might indicate the existence of multiple clones harboring different TCR rearrangements within the tumor. This is consistent with the ClonEvol analysis result that TALL017 had 2 major subclones at relapse originated from the same ancestor clone (Figure 4A-D). We further investigated previously reported paired diagnosis-relapse T-ALL cases and found another \( \text{NOTCH1} \) "switching" case (T-ALL-H-S00285) out of 13 paired cases.\(^\text{26}\) In this case, 2 \( \text{NOTCH1} \) mutations (both HD-SNV) were detected at diagnosis. One in minor subclones had disappeared (VAF = from .12 to .0003) and the other HD-SNV in major clones was replaced (VAF = from .33 to .04) by newly expanded HD-SNV at relapse (VAF = .26), which was not detected at diagnosis (VAF = .0008).

Type 3 clonal evolution at relapse was observed in 1 case (TALL018). In this case, the same gene mutations were observed in both diagnosis and relapse samples (Figure 3I-L). Insufficient treatment of T-ALL at diagnosis was considered to induce expansion of the same clone in this type of relapse. In this case, relapse occurred within 10 months of diagnosis. TALL018 was treated without MRD-based protocol. This case was assumed to remain MRD positive after remission induction. The mean VAF of all validated mutations in the corresponding normal sample was above \( 10^{-3} \) level at the end of the induction (mean VAF = .0026; Figure S9B).
4 | DISCUSSION

Based on the results of clonal analysis of samples at diagnosis and relapse, we identified NOTCH1 "switching" characterized by different NOTCH1 mutations in a major clone between diagnosis and relapse samples. This NOTCH1 "switching" has been previously reported using WAVE chromatograms. 38 In chronic lymphocytic leukemia (CLL), ultra-deep next-generation sequencing demonstrated similar changes of TP53 mutations in relapse and re-relapse samples. 39 Dysfunction of TP53 is considered the major cause of genomic instability in CLL cells, leading to resistance to treatment. 40,41 Taken together, "switching" of NOTCH1 mutations indicates that NOTCH1 plays an important role in both leukemogenesis and progression. According to previous studies, 10,42 increased luciferase activity by HD-SNV and PEST domain mutations seemed to be different depending on the position of mutations. These HD-SNV and PEST domain mutations were recognized as a weak tumor initiator even when they existed in cis, because these mutations failed to efficiently initiate T-ALL in mice. In contrast, they were reported to accelerate T-ALL with other driver mutations such as KRAS (detected in "switched" TALL023 sample), and they showed addiction to NOTCH1 signaling. Thus, we estimate that functional advantage in replacement of NOTCH1 mutations (HD-SNV to HD-SNV in TALL017 and UTR to PEST in TALL023, respectively) in "switched" T-ALL cases itself was not big, but the existence of NOTCH1 mutations in the "switched" clone might be important for selection advantage in relapse as the secondary event. Furthermore, we identified the acquisition of NT5C2 and BRAF mutations in cases of "switched" clones, respectively. NT5C2 mutation involves in clonal evolution during disease progression and relapse with induction of resistance to 6-mercaptopurine. 8,9,43 Mutations in MAPK pathway, including BRAF, were frequently observed in LCH. 44 Acquisition of these mutations in "switched" clones might be associated with selective advantage during tumor clonal evolution.

All 3 cases representing NOTCH1 "switching" or expansion at relapse contained the PEST alteration in the major clone. In TALL023, the case of NOTCH1 "switching," the PEST alteration at relapse was enriched from minor populations (VAF = from .07 to .42), and the UTR-specific mutation was eradicated (VAF = from .19 to 0). This suggests that NOTCH1 pathway activation via PEST alterations might offer a selection advantage in this case. In CLL, NOTCH1 mutations occur almost exclusively in PEST and are associated with poor prognosis. 45 Furthermore, PEST alterations were predominantly detected in diagnosis samples of relapsed cases rather than in non-relapsed cases, although this enrichment of PEST alterations in relapse cases was not confirmed in TARGET data. These results imply that PEST alterations are involved in the progression and early relapse of T-ALL. However, further research is warranted to decide the impact of PEST alterations in T-ALL.

The limitations of this study include its small sample size, treatment without MRD-based protocol, and its retrospective design in a single facility, leading to a selection bias. Nonetheless, mutations detected in this study using WXS of 30 T-ALL cases are consistent with previous reports. 6,7,46,47 Recent improvement in survival might be largely due to MRD-based risk stratification. Thus, further analysis is required to confirm the relevance of PEST mutations in the progression to relapse using large cohorts with or without MRD-based protocol.

In conclusion, we described that "switching" of NOTCH1 mutations demonstrates the importance of NOTCH1 signaling activation not only in the development but also in the progression of T-ALL. In
FIGURE 3  Depictions of clonal evolution from diagnosis to relapse in each relapse type. A-D, Clonal analysis in the representative case (TALL001) of relapse type 1, which acquired additional mutations at relapse in addition to all detected mutations at diagnosis. E-H, Clonal analysis in the representative case (TALL031) of relapse type 2. Several mutations presented in the major clone at diagnosis were lost, and additional mutations were acquired at relapse. I-L, Clonal analysis of relapse type 3. Same gene mutations were observed at both diagnosis and relapse in type 3 samples. A,E,I, Observed VAF of each mutation are shown in diagonal plots. Red dots indicate NOTCH1. B,F,J, VAF of detected mutations in each clone are indicated. C,G,K, Schematic description of population in each clone at diagnosis and relapse is shown. D,H,L, Schematic description of clonality in each clone at diagnosis (D) and relapse (R) is shown in phylogenetic trees. Detected mutations in each clone are listed. VAF, variant allele frequency.
addition, mutations in the PEST domain of NOTCH1 may be involved in T-ALL relapse in pediatric cases. Our results might be helpful for developing a new anti-NOTCH1 therapeutic strategy for refractory or relapsed T-ALL patients, although further studies are warranted.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.K. and J.T. wrote the manuscript; S.K., M.S., M.A., K.K., T.I., A.M. and Y.H. collected and analyzed data; S.K., M.S. and K.Y. performed experiments; Y.S. and S.M. developed bioinformatics pipelines; M.K., A.O., S.O. and J.T. gave conceptual advice; J.T. designed the study. All authors read and approved the final manuscript.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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