Detection of the TNFSF members BAFF, APRIL, TWEAK and their receptors in normal kidney and renal cell carcinomas

Vassiliki Pelekanou1, George Notas3, Katerina Theodoropoulou4, Marilena Kamp1, Dimitrios Takos5, Vassilia-Ismini Alexaki1, Jelena Radovic1, Frank Sofra3, Andreas Tsapis1, Efstathios N. Stathopoulos1,∗ and Elias Castana

1Laboratory of Experimental Endocrinology, School of Medicine, University of Crete, Heraklion, Greece
2Department of Urology, School of Medicine, University of Crete, Heraklion, Greece
3Department of Pathology, School of Medicine, University of Crete, Heraklion, Greece
4Inserm, U976, Paris, France; Université Paris-Descartes, Paris, France

Abstract. In advanced renal cell carcinoma (RCC), surgery combined with systemic chemotherapy and immunotherapy have had limited effectiveness. Therapeutic modalities targeting VEGF, PDGF, and c-kit using tyrosine kinase inhibitors and m-TOR using specific biologic factors are in development. Therapeutic approaches targeting TNF-alpha have shown limited efficacy, while anti-TRAIL (TNFSF10) antibodies have shown enhanced activity. The presence and potential significance of other members of the TNFSF has not been investigated. Here, we assayed the TNFSF members APRIL, BAFF, TWEAK and their receptors (BCMA, TACI, BAFFR, Fn14) in 86 conventional type clear cell RCC, using immunohistochemistry and correlated our findings with histological data and, in a limited series, follow-up of patients. We observed a differential expression of these TNFSF ligands and receptors in cancerous and non-cancerous structures. BAFF was found in all RCC; APRIL expression is associated with an aggressive phenotype, correlating negatively with patients’ disease-free survival, while TWEAK and its receptor Fn14 are heterogeneously expressed, correlating negatively with the grade and survival of RCC patients. This is the first study, presenting together the TNFSF members APRIL, BAFF, TWEAK and their receptors in different areas of normal renal tissue and RCC, suggesting a potential role of these TNFSF members in renal tumor biology.

Keywords: Renal cell carcinoma conventional type, tumor necrosis factor superfamily members (BAFF, APRIL, TWEAK), tumor necrosis factor receptor superfamily members (BCMA, TACI, TWEAK, Fn14)

1. Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and 95% of kidney neoplasms [11]. Beyond early stages, when surgery can be curative, treatment comprises of cytoreductive surgery and systemic chemotherapy [22], or immunotherapy [34], both proven modestly effective. However, recent studies suggest that targeting VEGF, PDGF, c-kit with tyrosine kinase inhibitors, or m-TOR, with specific biological factors represents a valid therapeutic approach in RCC [6, 7, 26–28, 36]. In this respect, exploring novel biological characteristics and factors in RCC might provide clues about new, selective therapeutic approaches of the disease.

TNFα has been detected in RCC [23]; however, anti-TNFα antibodies were of limited efficacy [13, 20]. In contrast, antibodies against TRAIL (another member of the TNFSF; TNFSF10) displayed enhanced activity [37]. Nevertheless, other members of this
superfamily (composed of 19 ligands and 29 receptors) have not yet been studied in this malignancy, such as BAFF (TNFSF13B), APRIL (TNFSF13) and TWEAK (TNFSF12). BAFF and APRIL have been implicated in the development and differentiation of B-lymphocytes, but they have also been identified in solid tumors, where they promote their development and progression [33]. TWEAK, on the other hand, is associated with a broad spectrum of cellular functions (from cell proliferation/differentiation to apoptosis) [39] and was also identified in immune-related nephritis [24].

In the present work, we identified by immunohistochemistry, the expression of APRIL, BAFF, TWEAK and their receptors (BCMA, TACI, BAFFR, TACI, TNFRSF17, TNFRSF13B, TNFRSF13C, TNFRSF12A respectively) in a series of 86 conventional clear cell RCCs. Our results show a differential expression of these ligands and receptors in normal and cancerous areas, related with the tumor phenotype and patients’ survival.

2. Materials and methods

2.1. Tissue specimens

One hundred nephrectomy specimens (histological reports and Hematoxylin-Eosin (H&E) stained slides) with the diagnosis of common or conventional (clear cell) renal cell carcinoma (RCC-CT) [16] were retrieved at random from the files of the Pathology Department of the University Hospital of Heraklion, out of a whole series of 214 diagnosed tumors. Fourteen (14) cases were excluded of further study for different reasons (partial nephrectomy specimens, incomplete macroscopical pathological data, technical reasons, etc.); only radical nephrectomy specimens were finally included. H&E-stained slides were reviewed and the tumor grade according to Fuhrman nuclear grading system [8] was reevaluated; nuclear grading was based on the highest-grade tumour component identified in the tumor [18].

Respective patients were operated at the Department of Urology, University Hospital of Heraklion, between September 1992 and November 2009, for a clinically diagnosed RCC. Of the eighty-six patients finally studied, 60 were men and 26 women. Their demographic and pathological data are presented in Supplementary Table 1. The slides were reviewed by two investigators independently and blindly to previous pathologic diagnoses and follow-up data. In case of disagreement, regarding nuclear grade, the final decision was made in consensus. Histological Fuhrman’s nuclear grading [8] revealed 14 grade 1 cases (7 male, 7 female) and 44 grade 2 cases (31 male, 13 female), 23 grade 3 cases (17 male, 6 female) and 5 grade 4 cases (5 male, 0 female). In fourteen patients (denoted in bold in Supplementary Table 1), 2 cm³ of the tumor and 2 cm³ of normal appearing tissue, away from the tumor mass, were harvested right after excision, under the guidance of a pathologist, immediately frozen in liquid nitrogen, and stored at −80°C, until processed for RNA isolation. The study had the approval of the University Hospital Research and Ethics Committee.

2.2. Immunohistochemistry

Eight tissue slides (3 μm) were cut serially from formalin-fixed and paraffin-embedded tissue sections of tumors, one for H&E staining and seven for specific immunostaining for BAFF, APRIL, TWEAK, BAFF-R, BCMA, TACI and Fn14.

After deparaffinization, slides were incubated with primary antibodies (30 min) to the different ligands and receptors. Specific antibodies used were: APRIL (hAprily-8 mouse monoclonal antibody, 1/100 dilution, ALX-804-149, Alexis Co, Lausanne, Switzerland), BAFF (ALX-804-131 monoclonal antibody/Buffy-2 clone, dilution 1/100, Alexis), TWEAK (sc-12405 goat polyclonal antibody, 1/100 dilution, Santa Cruz Biotechnology, CA, USA), BCMA (Vicky-1 rat monoclonal antibody, 1/100 dilution, ALX-804-151, Alexis), TACI (IMG-249 rabbit polyclonal antibody, 1/150 dilution, Imgenex, San Diego, CA, USA), BAFF-R (goat polyclonal, AF1162, dilution 1/100, R&D Systems, Minneapolis, MN, USA) and Fn14 (mouse monoclonal, sc-56250, dilution 1/50, Santa Cruz).

The UltraVision LP Detection System (TL-060-AL, Thermo Fisher Scientific, Fremont, CA) with Fast Red as chromogen was used for the detection of BAFF, APRIL and TACI, the DAKO K-06699 LSAB+ was used for the revelation of BAFF-R, while the K-1500 CSA kits (DAKO, Glostrup, Denmark) was used for immunodetection of BCMA, TWEAK and Fn14. Counterstaining was performed using Mayer’s hematoxylin. Known positive and negative controls (omission of the primary antibody) were used in every run, as previously described [1]. Quantification of immunohistochemical data was performed according
to the Allred scoring system [14]. Briefly, a proportion score (PS) was calculated representing the estimated proportion of positive tumor cells, as follows: none = 0; 1/100 of cells = 1; 1/10 of cells = 2; 1/3 of cells = 3; 2/3 of cells = 4; and 1/1 (i.e., all of the tumor cells are stained) = 5. An intensity score (IS) was also assigned, estimating the average staining intensity of positive tumor cells as follows: negative = 0; weak = 1; intermediate = 2; and strong = 3. The PS and IS was added to obtain a total score (range 0–8).

2.3. Quantitative RT-PCR

One µg of total RNA (isolated with Trizol, Invitrogen) was subjected to Thermoscript RT-PCR (Invitrogen), using primers shown in Supplementary Table 2 and synthesized by VBC Biotech (Vienna, Austria). Real-time PCR was performed with DyNAmo SYBR Green qPCR Kit with ROX (Finnzymes, Oy, Finland), using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction conditions for real-time PCR were 95°C for 3 min followed by 40 cycles of 95°C for 15 sec then 60°C for 60 sec. Changes were normalized according to Cyclophylin A expression.

2.4. Analysis of published gene-array data

Published gene array data that included expression analysis of RCC compared to normal kidney were used. We identified in the GEO datasets database (NCBI) dataset GSE781 [17], GSE6344 [10] and GSE15641 [15] that included gene array data from 50 RCC biopsies compared to 41 normal kidney biopsies. The control (normal kidney) and RCC gene-array data were extracted and analyzed.

2.5. Statistical analysis

Statistical analysis was performed with the SPSS, v18 (Chicago, IL). Microarray data were analyzed with the Genespring GX V11.0 (Agilent Technologies, Santa Clara, CA, USA), using the appropriate parametric or non-parametric tests. Statistical significance was set to \( p < 0.05 \).

3. Results

3.1. Immunohistochemical determination of BAFF, ARPIL, TWEAK, and their receptors (BAFF-R, TACI, BCMA and Fn14) in normal kidney and renal cell carcinoma

The expression of the TNFSF ligands and receptors in normal-appearing renal tissue is presented in Fig. 1. BAFF exhibited an intermediate-intensity staining of renal tubules, while rare cells were stained within Malpighian corpuscles. In contrast APRIL and TWEAK stained heavily renal tubules, while Malpighi...
bodies were negative. Both ligands' staining displayed a fine granular cytoplasmic pattern (Fig. 1, magnification). BAFF staining was homogeneous in the cytoplasm, with more intense pericellular staining, as, in contrast to APRIL, BAFF is not processed intracellularly, but is instead released from the cell surface, where it appears as a membrane-anchored protein [29, 30].

The staining of BAFF/APRIL and TWEAK receptors (BAFFR for BAFF, BCMA and TACI for BAFF/APRIL and Fn14 for TWEAK) presents equally discrete patterns: neither BAFFR, nor BCMA were detected. In contrast, TACI stained renal tubules in a homogeneous manner, while Fn14 staining exhibited a heterogeneous distribution; Malpighi bodies were constantly negative for all TNFSF receptors.

Figure 2 presents the differential expression of the TNFSF ligands and receptors in selected cases of RCC-CT, with different Fuhrman's nuclear grade. As shown, BAFF presented a homogeneous staining in all cases, independent of tumor grade, in accordance with previous findings [4]. In contrast, APRIL expression is increased with tumor nuclear grade. BAFF immunoreactivity was detected in the cytoplasm, while in some cases a more intense perinuclear staining was observed; the periphery of the cells was more heavily stained. BCMA was constantly absent, while the antibody to BAFFR stained rare cells in the tumor mass, independently of nuclear grade. In addition, TACI presented a moderate, grade-related, staining. TWEAK and its receptor, Fn14, presented a parallel expression in RCC-CT, suggesting an auto/paracrine mode of action: while negative in grade 1 tumors, an increase of staining was observed, related to the tumor grade. However, maximal staining for TWEAK was observed in grade 3 tumors, while for Fn14 in grade 4 RCC-CT. For both ligands, a heterogeneous staining pattern, with negative, moderate or high intensity areas, was observed. Staining of the TNFSF ligands and receptors was quantified by calculating the Allred score [14].

Comparison of staining for the different parameters (presented in Supplementary Table 1, as Allred scores) revealed a significant correlation of APRIL with TACI (rho = 0.431, p < 0.0001), TWEAK (rho = 0.457, p < 0.0001) and Fn14 (rho = 0.489, p < 0.0001) and of TWEAK with Fn14 (rho = 0.804, p < 0.0001). Finally TACI correlated with TWEAK and Fn14 (rho = 0.513 and 0.548, p < 0.0001 for both TWEAK and Fn14). These correlations are indicative of a parallel expression of the couples APRIL/TACI and TWEAK/Fn14 and of a possible role of these TNFSF ligands and their receptors in renal cancer biology.

In conclusion, our data suggest that RCCs-CT express the TNFSF ligands BAFF (homogenously) and APRIL and TWEAK (in a tumor Fuhrman's grade-related manner), and their receptors TACI and Fn14, while BAFFR and BCMA are not expressed. Furthermore, TWEAK and its receptor Fn14 were expressed heterogeneously in the tumor mass. Normal-appearing Malpighi bodies were constantly negative, while normal tubules express also BAFF, APRIL and TACI homogenously and the couple TWEAK/Fn14 in a heterogeneous manner.

3.2. Correlation of TNFSF ligands and receptors with patients’ survival

In a limited number of cases (46/86) we had additional clinical features, as described in Supplementary Table 1. Table 1 presents the non-parametric correlation of Allred scores for the different TNFSF ligands and receptors with clinical features. As shown, low TWEAK and/or Fn14 are related to a better prognosis of patients. This is further verified by the negative correlation of TWEAK and Fn14 with patients' overall and disease-free survival, a result equally found for the couple APRIL/TACI and the positive correlation of these molecule expression with the Fuhrman's tumor grade. No correlation with the therapy regiments was found, probably due to the small number of cases in each category.

Performing a ROC-curve analysis, we have established as a cut-off level of 5 in Allred score (meaning in most cases 30% of cells presenting an intermediate staining) for APRIL, TACI, TWEAK and Fn14 (the parameters which expressed a significant correlation with clinic pathological parameters). A univariate Kaplan-Meyer analysis (Fig. 3) revealed that APRIL and TWEAK Allred scores were significantly related to overall survival, while only APRIL was related to the disease-free survival of patients. This was further verified in a Cox regression multivariate model, in which only APRIL was retained as a significant variable in a backward stepwise model, related to the disease-free survival with a −2 Log Likelihood value of 259.238 (Chi square = 7.449, p < 0.006) and TWEAK was related to the overall survival of patients (−2 Log Likelihood 69.512, Chi-square = 13.191, p < 0.0001).
Fig. 2. Detection of BAFF, APRIL, TWEAK and their receptors (BAFFR, TACI, BCMA and Fn14) in selected cases of RCC-CT. TNFSF ligands and receptors were assayed as described in Materials and methods. Cases of Fuhrman Grades 1–4 are presented. Bar = 100 μm.
Table 1
Non-parametric correlation coefficient of clinicopathological features of tumors with the immunohistological scores for the different TNFSF ligands and receptors. Only statistically significant coefficients and one-tailed statistical significance are presented.

| Status (1 = Life, 0 = Dead) | Correlation | Coefficient signif. (p < ) |
|----------------------------|-------------|---------------------------|
| APRIL Score                | −0.344      | 0.010                     |
| TACI Score                 | −0.261      | 0.040                     |
| | N                        | 46          | 46                        |
| OS (months)                | −0.304      | 0.003                     |
| Correlation                | −0.340      | 0.001                     |
| Coefficient signif. (p < ) | 0.447       | 0.035                     |
| | N                        | 46          | 46                        |
| DFS (months)               | −0.420      | 0.002                     |
| Correlation                | −0.318      | 0.016                     |
| Coefficient signif. (p < ) | −0.453      | 0.001                     |
| | N                        | 46          | 46                        |
| Fuhrman nuclear Grade      | 0.537       | 0.001                     |
| Correlation                | 0.585       | 0.001                     |
| Coefficient signif. (p < ) | 0.909       | 0.001                     |
| | N                        | 86          | 86                        |

3.3. Detection of mRNA transcripts of BAFF, APRIL, TWEAK and their receptors in RCC and non-cancerous renal tissue (Fig. 4)

The expression of the TNFSF ligands and receptors in renal tissue is complicated, with some elements (ex. renal tubules) expressing them with a variable intensity, while Malpighi bodies and interstitial tissue being negative; tumors expressing equally differentially these molecules. In this respect, analysis of total RNA from these structures might provide less accurate results than immunohistochemistry. This was verified here, in a small subset of tumors (14/86, denoted in bold in Supplementary Table 1) in which fresh-frozen tissue, including areas of tumor, as well as areas of non-tumoral tissue was available, in addition to paraffin-embedded tumor blocks. As presented in Supplementary Table 1, 2/14, 12/14, 0/14, 5/14 and 4/14 tumors expressed an Allred score > 5 for BAFF, APRIL, TACI, TWEAK and Fn14 respectively. Results are presented as the differences of cancer versus normal tissue expression of the same patient. All parameters exhibited a decreased expression in cancer, as compared to normal tissue, with the notable exception of Fn14 mRNA, which increased in cancer tissue, independently of the Allred score of the tumor for the same parameters. However, a high dispersion of differences was observed, verifying our hypothesis (Fig. 4A).

In addition, we have re-analyzed 3 gene-array datasets, including data from 50 RCCs compared to non-cancerous kidney tissue (Fig. 4B). We have verified the increased Fn14 and decreased TACI mRNA expression in RCCs. Here too, changes in BAFF, APRIL and BAFFR were not significantly different from control values.

4. Discussion

BAFF, APRIL, TWEAK and their receptors have been initially considered as exclusive elements of the immune system, regulating the differentiation and fate of lymphocytes [3, 19], being also involved in immune-related diseases. Recently, they have also been identified in normal and pathological tissues [2], including various malignancies [25, 32, 35]. In renal tissue, BAFF was found up-regulated in areas of RCCs, proposed as an element of the tumor immune-escape strategies [4]. Its expression was attributed to infiltrating monocytes, implicated in increased proliferation of different renal cell types, regulating the expression of different proinflammatory molecules [9]. TWEAK, on the other hand, was identified in immune-related nephritis [24], while its expression in a wide variety of different renal cell types, parallels the wide variety of apparently conflicting cellular responses, ranging from proliferation to cell death. TWEAK has also been recognized in many tissue-resident progenitor cells, and a role of it in tissue regeneration has been proposed [5], while it has been implicated in promotion of angiogenesis, increase of the neurovascular unit permeability and the regulation of precursor cell differentiation [2]. Concerning APRIL, no data exist in renal tissue. APRIL, in contrast to many other TNFSF ligands, is a proliferation-promoting factor of cells in tissue culture [12]. Indeed, shRNA targeting APRIL was found to suppress pancreatic cancer cell growth [38]; furthermore APRIL was reported to increase pro-
Fig. 3. Kaplan-Meyer survival curves for APRIL and TWEAK, in RCC. Curves were generated, based in 46 cases, in which overall and disease-free survival was present. A cut-off value of 5 in Allred score was applied, based on a preliminary ROC curve analysis. Right curves present data for which Allred score was >5, while left curves show cases for which Allred score was ≤5. Table presents a Kaplan-Meyer survival analysis of data. Bold characters point out statistically significant relations.

|          | OS  | DFS |
|----------|-----|-----|
|           | Chi square | Significance | Chi square | Significance |
| APRIL Score | 7.559 | 0.006 | 7.488 | 0.006 |
| TACI Score  | 1.266 | 0.261 | 0.981 | 0.322 |
| TWEAK Score | 3.567 | 0.05  | 3.131 | 0.0695 |
| Fn14 Score  | 0.269 | 0.204 | 0.247 | 0.620 |

In contrast to previous studies, in which upregulation of APRIL in cancer lesions was attributed to infiltration of APRIL-producing neutrophils [21], recent data, as well as results presented here, suggest that tumor cells may also produce and secrete APRIL. Indeed, high expression of APRIL was detected in human colon and thyroid cancers [12], as well as in adipose tissue-derived tumors [1].

In the present study, we have analyzed, by immunohistochemistry, a series of 86 RCCs of the conventional type. We have revealed specific patterns of ligands and receptors expression in non-cancerous and cancerous tissue: in normal appearing renal tissue, Malpighi bodies were constantly negative for all ligands and receptors, with the exception of BAFF, which stained very rare cells of unknown origin (constitutive cells of the Malpighi body or infiltrating leucocytes). In contrast, normal tubules were stained moderately for BAFF and APRIL, while TWEAK was stained heavily, but heterogeneously, for its receptor Fn14. It is plausible therefore that in normal renal tissue, BAFF and APRIL might signal exclusively through TACI.

In contrast, in areas of RCC-CT a moderate expression of BAFF and APRIL was observed (the former being independent, while the latter related to the tumor proliferation of hepatocellular carcinoma cells [31].
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Fig. 4. Detection of normalized mRNA expression of BAFF, APRIL, TWEAK and their receptors (BAFFR, TACI, BCMA and Fn14) in renal cell carcinoma, by qRT-PCR. A) Normalized qRT-PCR differences between cancerous and normal renal tissue (1 = normal). Table presents means and statistical values of qRT-PCR difference of expression of the TNFSF ligands and receptors in fresh-frozen tissue samples of a subset of cases (n = 14, presented in bold in Supplementary Table 1). B) Expression of TNFSF ligands and receptors in three published arrays (accession numbers are given in the legend).

grade), while TACI was equally expressed moderately, and BAFFR immunostaining emerged similarly, although at a low, grade-independent manner. In addition, comparing the expression of ligands and receptors during the evolution of the disease (as expressed by the Fuhrman nuclear grade), BAFF expression was constant, while APRIL immunochemical score increased with the increased nuclear grade of the disease. It is therefore plausible that BAFF (considered here as a trophic factor, as in the majority of normal and cancerous tissues) might additionally signal primarily through BAFFR, while both ligands might interact with TACI. Of special interest is that APRIL, considered as a proliferation-promoting factor in vitro [12], is preferentially expressed in high-grade tumors, which are more aggressive, displaying a higher proliferation potential, a feature previously reported in breast cancer [32] and adipose-tissue derived tumors [1]. APRIL is reported here as the parameter related to disease-free survival of patients. However, these results should be verified in larger series of data. Finally, we have revealed that the couple APRIL/TACI is constantly correlated with the TWEAK/Fn14, suggesting a parallel way of action of these two TNFSF ligands in renal cell carcinomas.

In a recent report, TWEAK was exerting a proliferative action in different renal cell types (kidney mesangial cells, podocytes and tubular cells), regulating also the expression of inflammatory factors [9]. Our findings confirm the increased expression of Fn14 in RCC-CT; however, an increased (albeit heterogeneous) staining of normal and tumoral tissue areas by TWEAK/Fn14 suggests a potential role of this couple in normal renal tissue and RCC-CT biology. Of further potential importance is the observed negative correlation of TWEAK (which might produced by renal cells per se, as we have not observed any significant area of inflammation) with patients’ survival. Interestingly, in a recent report, renal cell expression of Fn14 was correlated with accentuated nephritis [24]. The heterogeneous staining of TWEAK/Fn14, moreover, in normal renal tissue might be an early sign of renal distress.

In conclusion, our data suggest that renal parenchyma expresses in a differential way BAFF, APRIL, TWEAK and their receptors. The above TNFSF members are further expressed in RCC, correlating with the evolution of the disease (APRIL/TACI and TWEAK/Fn14), suggesting a potential role in RCC tumor biology and kidney physiology. This is another example of the production of immune-related molecules by solid tumors and relating them to the evolution of the disease.

Acknowledgements

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Supplementary Table 1

| Clinicopathological Data of analyzed cases |
|------------------------------------------|
|                                          |

- [Table Content]
| No | Sex | T2A | Clinical Stage | ON | UPS | Ovaria | Radio | Hist. Factor | Grade | HIF2 Score | NFkB Score | HIF2 Score | NFkB Score | HIF2 Score | NFkB Score |
|----|-----|-----|----------------|----|-----|--------|-------|-------------|-------|------------|------------|------------|------------|------------|------------|
| 1  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 2  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 3  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 4  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 5  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 6  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 7  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 8  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 9  | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 10 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 11 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 12 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 13 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 14 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 15 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 16 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 17 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 18 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 19 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 20 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 21 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 22 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 23 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 24 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 25 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 26 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 27 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 28 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 29 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 30 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 31 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 32 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 33 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 34 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 35 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 36 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 37 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 38 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 39 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |
| 40 | 1   | 1   | 3              | 3  | 0   | 0      | 0     | 0           | 0     | 0          | 0          | 0          | 0          | 0          | 0          |

**Supplementary Table 1 (Continued)**
References

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