ORIGINAL RESEARCH

Risk of squamous cell skin cancer after organ transplant associated with antibodies to cutaneous papillomaviruses, polyomaviruses, and *TMC6/8 (EVER1/2)* variants

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Abstract

Squamous cell skin cancer (SCSC) disproportionately affects organ transplant recipients, and may be related to increased viral replication in the setting of immune suppression. We conducted a nested case–control study among transplant recipients to determine whether SCSC is associated with antibodies to cutaneous human papillomaviruses (HPV), to genes associated with a rare genetic susceptibility to HPV (*TMC6/TMC8*), or to human polyomaviruses (HPyV). Cases ($n = 149$) had histologically confirmed SCSC, and controls ($n = 290$) were individually matched to cases on time since transplant, type of transplant, gender, and race. All subjects had serum drawn immediately prior to transplant surgery. Antibodies to 25 cutaneous HPVs and six HPyVs were assayed by detection of binding to virus-like particles, and 11 TMC6/8 variants were genotyped. After correction for multiple comparisons, only antibodies to HPV37 were associated with SCSC (OR 2.0, 95% CI 1.2–3.4). Common genetic variants of TMC6/8 were not associated with SCSC, but three variants in *TMC8* (rs12452890, rs412611, and rs7208422) were associated with greater seropositivity for species 2 betapapillomaviruses among controls. This study suggests that some betaHPVs, but not polyomaviruses, may play a role in the excess risk of SCSC among transplant recipients.

Introduction

Most solid organ transplant recipients take maintenance immunosuppressant medications to prevent graft rejection, and therefore, have increased susceptibility to viral infections and excess risk of virus-related cancers. A large registry-based study of transplant recipients and cancer risk [1] reported highly elevated standardized incidence ratios (SIRs) associated with virus-related cancers. Examples include Kaposi sarcoma, linked to human herpesvirus type 8 infection (SIR 61), liver cancer often caused by

hepatitis B or C viruses (SIR 11), non-Hodgkin lymphoma, associated with Epstein–Barr virus (SIR 11). In contrast, cancers not associated with viruses, such as breast and prostate cancer, do not occur in excess among transplant recipients [1].

The most commonly occurring malignancy posttransplant is nonmelanoma skin cancer, and immunosuppressive medications and prior sun exposure are important in their etiology [2, 3]. Although prior studies have reported very high, >50-fold, increased risks for squamous cell skin cancer (SCSC) among transplant recipients compared to

the general population [4, 5], no definitive viral etiology has been established for SCSC [6]. Transplant physicians and dermatologists prioritize prevention and early detection efforts to avoid SCSC that occurs in excess after transplant. Even so, some transplant recipients develop aggressive SCSC with early onset, multiple tumors, and high recurrence rates [7, 8].

Specific types of SCSC have been definitively associated with two viruses, human papillomaviruses (HPV) and human polyomaviruses (HPyV). Cutaneous HPV types were initially associated with SCSC because genus beta-papillomavirus (betaHPV) were detected in skin tumors from individuals with a rare genetic disorder [9, 10] attributed to mutations in the TMC6/8 (EVER1/2) genes [11]. The TMC6/8 proteins may act as barriers to HPV transcription factors and replication, with their loss leading to susceptibility to HPV-related skin lesions [12]. However, no high-risk HPV type or subset of types has been consistently associated with SCSC in transplant recipients [13–15].

The Merkel cell polyomavirus [16] (MCPyV) is strongly associated with a rare cutaneous cancer, Merkel cell carcinoma [17]. There are six cutaneous human polyomaviruses (HPyV: MCPyV, JCPyV, KIPyV, WUPyV, HPyV6, and HPyV7) that have cutaneous pathogenic potential [18]. Recent studies provided mixed results concerning the possibility that HPyV [18–20] or HPV [13, 21] is associated with SCSC among transplant recipients. In this study, we investigated whether antibodies to cutaneous HPyV or HPV, or genetic variation in TMC6/8, were associated with SCSC among highly susceptible transplant recipients.

Materials and Methods

Study population

This nested case–control study included participants in the Skin Cancer after Organ Transplant (SCOT) cohort study [22]. The SCOT cohort includes ~2000 renal and cardiac transplant recipients who received their transplant in the Seattle area between 1995 and 2010. Participants resided in Washington, Wyoming, Alaska, Montana, or Idaho at the time of transplant. Exclusions included history of transplant prior to 1995, age less than 18 years old at time of transplant, inability to communicate in English, a history of SCSC prior to transplant, and graft failure within 3 months of transplant. Study procedures were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Participants were asked to report any skin condition that required a biopsy, and biopsy reports were reviewed to confirm a diagnosis of invasive SCSC. The first instance of

pathology-confirmed SCSC after transplant served as the case diagnosis date. Controls were participants without SCSC, matched to cases on year of transplant (±2 years), age at transplant (±5 years), time since transplant (exact months), transplant hospital, donor type (deceased or living), organ transplanted (kidney, kidney and pancreas, or heart), gender, and race (white or not white).

We enrolled 172 cases (87% of 198 confirmed SCSC by pathology report review) and 332 controls (80% of the 415 matched controls selected from the cohort) in the nested case–control study. There were eight participants initially identified as controls based on a prior mailed survey, who reported SCSC at the time of the in-person interview. These participants became cases for whom new controls were selected.

Interview

We collected detailed information during an in-person interview. In addition to history of biopsies and dermatologic visits, we asked questions about sun exposure history included skin type, amount of time spent outdoors, susceptibility to burning and tanning, use of tanning beds, and sun avoidance practices. We also asked about medical history, including type of immunosuppressive therapies, residence history, and ethnicity.

Biospecimens

Pretransplant serum samples were collected and stored at the time of transplant by the Puget Sound Blood Center. We retrieved and tested serum samples from 149 cases (86.6%) and 290 controls (87.3%). In addition, 90% of cases and 84% of controls agreed to a blood draw or saliva sample (OraGene; DNAGenotek, Kanata, ON, Canada) collection at the time of the interview, which served as the source of genomic DNA. There was an overlap in available serology data and DNA results data for 143 cases and 259 controls.

Serologic assays

The antibody detection method was based on glutathione-S-transferase (GST) capture enzyme-linked immunosorbent assay (ELISA) described by Sehr et al. [23]. in combination with fluorescent bead technology (Luminex, Austin, TX) [24]. The antibody bound to viral antigen was quantified via the median fluorescence intensity (MFI) of at least 100 beads of the same internal color on the Luminex platform.

HPV L1 DNA types that were cloned into the expression plasmids were received as gifts from the following colleagues: types 12, 14, 21, and 22 from G; Orth (Paris,

France), types 37 and 47 from E. M. de Villiers (Heidelberg, Germany); and HPV type 96 from O. Forslund (Malmö, Sweden). The other HPVL1 expression plasmids were from M. Pawlita (Heidelberg, Germany), who also provided plasmids for BKPyV and JCPyV. All L1 proteins were partially purified by ion exchange chromatography [25], which improved reactivity. HPyV6 and HPyV7 sequences were purchased from Addgene (Cambridge, MA). Cloning of MCPyV, WUPyV, and KIPyV plasmids was previously described [26].

Blood samples from cases and controls were tested for antibodies to 25 betaHPVs and six polyomaviruses. Laboratory personnel were blinded, and case and control samples were randomly ordered. Concordance of 10% replicates for the HPV panel, ranged from 0.70 to 0.96, and for the HPyV replicates ranged from 0.79 to 0.92. The cut point for a seropositive result was set at MFI > 500 for HPV and MFI > 5000 for HPyV antigens.

Genetic assay

Single-nucleotide polymorphisms (SNPs) were selected from the *TMC6* and *TMC8* region (ch 17: 76,108,999–76,139,049), plus 4000 base pairs 5′ and 3′ of the region, to maximally capture common genetic variation [27]. Selected SNPs (*n* = 12) had a minor allele frequency of at least 0.05% among Caucasians in the reference population (HapMap), and were genotyped using the VeraCode platform. One SNP from *TMC8* (rs17773854) was excluded from analysis, due to call proportion <0.90 and HWE *P* < 0.01. Approximately 7% quality control replicates were included in the assay. Replicate concordance across SNPs ranged from 94.1% to 100%, averaging 99.1%.

Statistical analysis

Odds ratios (OR) and 95% confidence intervals (CI) were generated by conditional logistic regression models. Further adjustment for age at reference (i.e., age at cancer diagnosis for cases and age at a comparable time point posttransplant for matched controls) was added to reduce possible residual confounding. History of UV exposure (susceptibility to burning or tanning and number of sunburns) or type of immunosuppressive drug (history of ever use of each of the following: azathioprine, cyclosporine, tacrolimus, mycophenolate mofetil) did not appreciably change the estimates and were not included in final estimates. To correct for multiple comparisons, we used a Holm–Bonferroni step-down procedure [28] to determine statistically significant estimates (corrected *P* < 0.05) for individual betaHPV types. Associations between variants and SCSC were computed assuming a log-additive conditional model.

Results

The distribution of matching factors for this nested case–control study is detailed in Table S1. Serum samples were collected immediately prior to transplant, and cases were diagnosed for SCSC with an average of 5.4 years (3.2 SD) after transplant. The majority of study participants were seropositive for HPyV6, KIPyV, MCPyV, and WUPyV (Table 1). The JCPyV assay had lower levels of positivity and the WUPyV had highest levels. There was no association between risk of SCSC and seropositivity to individual HPyV or grouped betaHPV types overall, multiple betaHPV types, or betaHPV phylogenetic species.

Individual results for the HPV assays and risk of SCSC are shown in Table 2. Among the betaHPV types assayed, antibodies to HPV37 occurred most commonly in those with SCSC (26.8% in cases vs. 17.6% in controls), and were associated with a significantly increased risk of SCSC (OR 2.0, 95% CI 1.2–3.4, *P* = 0.005). Elevated but

Table 1. Risk of squamous cell skin cancer among solid organ transplant recipients associated with antibodies to cutaneous viruses, Seattle area SCOT cohort 1995–2010.

	Controls (n = 290)		Cases (n = 149)			
	n	(%)	n	(%)	OR	(95% CI)
Polyomaviruses[1]						
JCPyV	47	22.7	16	14.5	0.8	(0.4–1.6)
KIPyV	146	70.5	78	70.9	1.1	(0.6–2.0)
MCPyV	131	63.3	63	57.3	0.7	(0.4–1.2)
WUPyV	172	83.1	99	90.0	1.2	(0.6–2.6)
HPyV6	137	60.1	65	52.0	0.6	(0.4–1.0)
HPyV7	85	37.3	57	45.6	1.2	(0.7–1.9)
BetaHPVs Types						
Seronegative	73	(25.2)	41	(27.5)	ref	
Seropositive	217	(74.8)	108	(72.5)	1.0	(0.6–1.5)
1 type	61	(21.0)	34	(22.8)	1.0	(0.6–1.8)
2 to 4 types	90	(31.0)	31	(20.8)	0.6	(0.3–1.1)
5 to 9 types	34	(11.7)	24	(16.1)	1.5	(0.7–3.0)
10+ types	32	(11.0)	19	(12.8)	1.2	(0.6–2.6)
Species 1[2]						
Negative	129	(44.5)	74	(49.7)	ref	
Positive	161	(55.5)	75	(50.3)	0.8	(0.5–1.3)
Species 2						
Negative	144	(49.7)	73	(49.0)	ref	
Positive	146	(50.3)	76	(51.0)	1.1	(0.7–1.7)
Species 3						
Negative	222	(76.6)	114	(76.5)	ref	
Positive	68	(23.4)	35	(23.5)	1.1	(0.7–1.8)

CI, confidence intervals; OR, odds ratios; SCOT, Skin Cancer after Organ Transplant.
[1]Polyomavirus serology performed on a subset of participants (*N* = 110 cases and *N* = 207 controls).
[2]Genus and species grouped according to phylogenetic description of de Villiers et al. [56].

nonsignificantly increased risks were associated with HPV15, HPV20, and HPV36. There was also an increased risk of SCSC associated with HPV1 (OR 1.9, 95% CI 1.1–3.1, $P = 0.042$), the common plantar wart virus.

In genetic analyses, we explored whether common variants in *TMC6/8* were related to betaHPV seropositivity (Table 3). Among controls, three variants in *TMC8* were associated with greater seropositivity for antibodies against betaHPV species 2: the G allele of rs12452890; the A allele of rs412611; and the A allele of rs7208422. We then assessed whether variant alleles in the TMC6/8 region were associated with risk of SCSC (Table S2), and found no increased risk of SCSC associated with these variants. There were suggestive, but not significant associations with two SNPs in TMC6 and reduced risk of SCSC (G allele of rs16970842 with OR 0.56, 95% CI 0.29–1.08, and G allele of rs7218589 with OR 0.53, 95% CI 0.27–1.04).

Table 2. Risk of squamous cell skin cancer among transplant recipients associated with HPV seropositivity, Seattle area SCOT cohort 1995–2010.

HPV type	Genus/ Species[1]	Controls (n = 290) n	(%)	Cases (n = 149) n	(%)	OR	(95% CI)	P[2]
HPV1	μ	137	(47.2)	82	(55.0)	1.6	(1.0–2.5)	0.042
HPV2	α	47	(16.2)	29	(19.5)	1.4	(0.8–2.5)	
HPV4	γ	156	(53.8)	79	(53.0)	1.1	(0.7–1.6)	
HPV8	$\beta 1$	54	(18.6)	27	(18.1)	1.0	(0.6–1.7)	
HPV9	$\beta 2$	71	(24.5)	32	(21.5)	0.8	(0.5–1.4)	
HPV12	$\beta 1$	63	(21.7)	36	(24.2)	1.2	(0.7–2.1)	
HPV14	$\beta 1$	46	(15.9)	26	(17.4)	1.2	(0.7–2.3)	
HPV15	$\beta 2$	44	(15.2)	30	(20.1)	1.6	(0.9–2.8)	
HPV17	$\beta 2$	44	(15.2)	24	(16.1)	1.3	(0.7–2.3)	
HPV20	$\beta 1$	45	(15.5)	32	(21.5)	1.6	(0.9–2.9)	
HPV21	$\beta 1$	50	(17.2)	30	(20.1)	1.2	(0.7–2.1)	
HPV22	$\beta 2$	30	(10.3)	16	(10.7)	1.3	(0.6–2.4)	
HPV23	$\beta 2$	27	(9.3)	14	(9.4)	1.2	(0.6–2.4)	
HPV24	$\beta 1$	52	(17.9)	22	(14.8)	1.0	(0.5–1.8)	
HPV36	$\beta 1$	37	(12.8)	26	(17.4)	1.6	(0.9–2.8)	
HPV37	$\beta 2$	51	(17.6)	40	(26.8)	2.0	(1.2–3.4)	0.005
HPV38	$\beta 2$	31	(10.7)	12	(8.1)	0.8	(0.4–1.9)	
HPV47	$\beta 1$	42	(14.5)	28	(18.8)	1.3	(0.8–2.2)	
HPV49	$\beta 3$	39	(13.4)	27	(18.1)	1.3	(0.8–2.3)	
HPV75	$\beta 3$	41	(14.1)	23	(15.4)	1.3	(0.7–2.3)	
HPV76	$\beta 3$	32	(11.0)	18	(12.1)	1.0	(0.5–1.9)	
HPV80	$\beta 2$	34	(11.7)	23	(15.4)	1.3	(0.7–2.3)	
HPV92	$\beta 4$	23	(7.9)	15	(10.1)	1.4	(0.7–2.9)	
HPV93	$\beta 1$	7	(2.4)	5	(3.4)	1.5	(0.4–5.5)	
HPV96	$\beta 5$	41	(14.1)	24	(16.1)	1.2	(0.7–2.2)	

CI, confidence intervals; HPV, human papillomaviruses; OR, odds ratios; SCOT, Skin Cancer after Organ Transplant.
[1]Species grouped according to phylogenetic description of de Villiers et al. [56].
[2]P-values are corrected for multiple comparisons.

Table 3. Proportion of transplant recipients controls seropositive for betaHPV species[2] 1–3 by *TMC6/8* variants, Seattle area SCOT cohort 1995–2010.

Variant	Species 1 (N = 141) n	%	Species 2 (N = 129) n	%	Species 3 (N = 61) n	%
rs12452890						
AA	31	50.8	24	39.3	14	22.9
AG	76	56.7	63	47.0	32	23.9
GG	34	53.1	42	65.6[1]	15	23.4
rs16970842						
AA	113	53.8	105	50.0	53	25.2
AG/GG	28	57.1	24	49.0	8	16.3
rs16970849						
GG	130	54.9	123	51.9	58	24.5
GA/AA	11	50.0	6	27.3	3	13.6
rs2871647						
AA	109	51.2	101	47.4	48	22.5
AC/CC	32	69.6[1]	28	62.2	13	28.3
rs383603						
CC	81	52.9	78	51.0	33	21.6
CG	49	55.1	44	49.4	24	27.0
GG	11	64.7	7	41.2	4	23.5
rs412611						
GG	120	54.1	103	46.4	51	23.0
GA	21	56.8	26	70.3[1]	10	27.0
rs454138						
GG	54	56.8	50	52.6	27	28.4
GC	65	53.3	53	43.4	24	19.7
CC	22	52.4	26	61.9	10	23.8
rs7208422						
AA	39	59.1	41	62.1	15	23.4
AT	70	54.3	63	48.8	32	24.8
TT	32	50.0	25	39.1[1]	14	21.2
rs7218589						
CC	117	54.4	107	49.8	53	24.7
CG/GG	24	54.6	22	50.0	8	18.2
rs8068430						
TT	91	50.3	83	45.9	39	21.6
TC/CC	50	56.8	46	59.0	22	28.2
rs9807014						
CC	98	55.7	88	50.0	43	24.4
CT/TT	43	51.8	41	49.4	18	21.7

[1]χ^2 $P < 0.05$ for difference in seropositivity across genotypes.
[2]Genus betaHPV species described in Table 2.

Discussion

We measured cutaneous HPV and HPyV antibodies in samples collected immediately prior to transplant, before the impact of transplant-associated immunosuppression medications that might allow viral reactivation, to determine if antibodies detected prior to transplant were associated with later SCSC development. We found that a species 2 betaHPV type, HPV37, was significantly associated with a twofold increased risk of SCSC after transplant.

In addition, infection with species 2 betaHPV was associated with 3 TMC6/8 variants among controls.

We also examined the risk of SCSC associated with six HPyV, as MCPyV DNA has been detected in SCSC tumor tissue in some [18, 29] but not all studies [19, 30]. The lack of association with HPyV and most HPV in this study may indicate that unincluded or undiscovered cutaneous viruses may be associated with SCSC. Future studies may include previously unexamined cutaneous viruses to assess their influence on risk of SCSC.

Mucosal HPVs, specifically HPV16 and other high-risk types, have been recognized as a necessary causal factor in the majority of cervical, anogenital, and oropharyngeal cancers [31]. They are mechanistically central to the development and maintenance of HPV-related cancers. In contrast, betaHPV may be involved in the development but not maintenance of malignancy. For example, cutaneous HPVs are often absent or present in very low copy numbers in SCSC tumors but present at higher levels in precancers [32]. The E6 and E7 genes of some betaHPV types do appear to disrupt the p53 and pRb pathways in various ways, but less effectively than high-risk HPVs [33–36]. In addition, betaHPVs may interact with UV-damaged cells to induce SCSC by interfering with DNA repair [37–40], cell cycle control [41, 42], or increasing cell life span [39]. The betaHPVs may act as promoters of SCSC after UV damage through a combination of these pathways, and genetic damage from UV exposure and betaPV infections are acquired years before transplant.

We observed an increased risk of SCSC associated with antibodies to HPV1, which is associated with plantar warts in the general population [43]. This finding may suggest that transplant recipients who develop SCSC have a generalized susceptibility to some types of cutaneous HPV lesions. However, there was no increased risk of SCSC associated with antibodies to the two other cutaneous wart-causing cutaneous types (HPV2 and HPV4) measured.

Two prior case–control studies in transplant recipients [13, 21] have reported on the relationship between serologic antibodies to HPV and risk of SCSC. Casabonne et al. [21]. enrolled 140 cases and 454 controls from two British transplant centers and reported no associations between HPV seropositivity and SCSC risk, but did not include antigens for HPV37. Proby et al. [13]. included 210 cases and 394 control transplant recipients from multiple centers across Europe. They found borderline statistically significant associations between risk of SCSC and a combined measure of antibody/hair follicle HPV DNA positivity for three types (HPV5 OR 2.0, 95% CI 0.95–4.3; HPV24 OR 2.0, 95% CI 0.9–4.1; HPV36 OR 2.4, 95% CI 1.0–5.4). Our study did not confirm an association with HPV24 (HPV24 OR 1.0, 95% CI 0.5–1.8), but

did confirm the marginal association with HPV36 (OR 1.6, 95% CI 0.9-2.8). Proby et al. [13]. did report an increased prevalence HPV37 DNA in plucked eyebrow hairs of cases compared to controls; however, HPV37 antigens were not included in their serologic analysis. Thus, HPV36 and HPV37 were associated with SCSC in transplant recipients in both studies.

Other studies have reported on HPV serology and SCSC risk in immune-competent populations [44–49]. One found no association between HPV serology and risk of SCSC in a Swedish population [46], and another found an association with seropositivity to any HPV type among participants less than 50 years old [49]. Another recent European study reported on risk of recurrence of SCSC was associated with seropositivity to HPV15, 24, 60, and 95 [50]. Andersson et al. [51] conducted a nested case–control study of 633 matched pairs, and found an increased risk of SCSC associated with species 2 betaHPV types (OR 1.3, 95% CI 1.1–1.7). Although HPV37 is a member of species 2, it was not included in their study; this study supports the suggestion for a role for species 2 betaHPV in SCSC.

Ramoz et al. found [11] that individuals with a rare dermatologic condition, epidermodysplasia verruciformis (EV), have a high risk of warts and SCSC associated with mutations in TMC6/8. This mutation may increase susceptibility to HPV infection, particularly betaHPVs. Karagas et al. reported SCSC risk in a US study was associated with multiple cutaneous HPV types [48]. A subsequent study in the same population [52] found that a coding variant in TMC8, rs7208422, was associated with SCSC and betaHPV seropositivity among controls. This study found that rs7208422 was associated with seropositivity to species 2 betaHPV types among controls (Table 3). We also found that two other SNPs in TMC8 (rs12452890 and rs412611) were associated with seropositivity to species 2 betaHPVs. These findings add to the evidence that TMC mutations may influence the risk of species 2 betaHPV infections.

The study has several limitations and strengths. It is potentially limited by its sample size, which may miss modest associations. Another point to consider is that the controls were matched closely to cases, as described in the Materials and Methods section. Given that the prevalence of SCSC is high in this population, this close matching may have attenuated the effect estimates; however, matching might also be considered a strength of the study as immune response is likely a very important component of excess risk of SCSC in this population. A further limitation is that tumor virus status remains unknown, which would be a more definitive indication of a potential association between HPV37 and SCSC. Also, multiple comparisons are a potential source of false-positive results,

but suggestive findings concerning species 2 antibodies and SCSC in other studies support the HPV37 finding. A strength of the study is that it represents one geographic area, and therefore a homogenous group. Although the reproducibility of the antibody assay was good, the results may not accurately reflect prevalence of prior exposure to specific HPV types measured. For example, the low proportion of samples found to be seropositive for HPV38 was likely an underestimate based on prior literature in transplant recipients [13, 21, 53]. The nested case–control study approach and use of blood samples drawn before transplant make it unlikely that the exposure assessment was affected by immune suppression following transplant.

In summary, it is likely that relevant cutaneous betaHPV infections are acquired early in life and therefore prior to transplant, as demonstrated in family studies of betaHPV [54]. Specific variants in the TMC6/8 gene region may contribute to susceptibility to species 2 infections. If transplant recipients have latent cutaneous HPV infections that are re-activated in the posttransplant period, the infections may promote cell growth, increasing the likelihood that UV-damaged cells acquire additional genetic damage. With respect to HPV37, the increased risk remained significant after correction for multiple comparisons and warrants further study. No clinical role for this finding is warranted at this time. HPV37 was originally isolated from a subtype of SCSC, a keratoacanthoma [55], suggesting that it may also be found in other SCSC tumors. A prospective study design with repeated measures before and after immunosuppression is the next step toward testing the putative cutaneous virus-SCSC association.

Acknowledgments

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of SCOT Cohort study participants by matching variables.
Table S2. Risk of SCSC among transplant recipients related to *TMC6/8* variants.