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Multiomic Characterization and Molecular Profiling of Nuclear Protein in Testis Carcinoma

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ABSTRACT

PURPOSE Nuclear protein in testis carcinoma (NC) is an underdiagnosed and aggressive squamous/poorly differentiated cancer characterized by rearrangement of the gene *NUTM1* on chromosome 15q14. Co-occurring alternations have not been fully characterized.

METHODS We analyzed the genomic and immune landscape of 54 cases of NC that underwent DNA- and RNA-based NGS sequencing (Caris).

RESULTS While NC is driven by *NUTM1* fusion oncoproteins, co-occurring DNA mutations in epigenetic or cell cycle pathways were observed in 26% of cases. There was no significant difference between the fusion partner of *NUTM1* and co-occurring gene mutations. RNA sequencing analysis showed increased *MYC* pathway activity in NC compared with head and neck squamous cell carcinoma (HNSCC) and lung squamous cell carcinoma (LUSC), which is consistent with the known pathophysiology of NC. Characterization of the NC tumor microenvironment using RNA sequencing revealed significantly lower immune cell infiltration compared with HNSCC and LUSC. NC was 10× higher in patients with HNSCC and LUSC younger than 50 years than in those older than 70 years.

CONCLUSION To our knowledge, this is the first series of NC profiled broadly at the DNA and RNA level. We observed fewer intratumoral immune cells by RNA sequencing, which may be associated with anecdotal data of lack of immunotherapy benefit in NC. High *MYC* pathway activity in NC supports ongoing trials targeting *MYC* suppression. The incidence of NC among patients younger than 50 years with LUSC/HNSCC supports testing for NC in these patients. The prognosis of NCs remains dismal, and future studies should focus on improving the response to immunotherapy and targeting MYC.

ACCOMPANYING CONTENT

Appendix

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INTRODUCTION

Nuclear protein in testis (NUT) carcinoma (NC) is an underdiagnosed malignancy characterized by rearrangement of the gene *NUTM1* on chromosome 15q14.[1] NC presents as a poorly differentiated squamous cell carcinoma that often originates from the lungs or head and neck.[2] In approximately two thirds of NC cases, *NUTM1* on chromosome 15q14 is fused to *bromodomain* (*BRD*)-4 on chromosome 19p13.1. The remaining cases that lacked BRD4 rearrangement included *NSD3* (*WHSC1L1*) and *BRD3* and rarely *ZNF532* and *ZNF592*.[3]

In 1999, the first case of NC was described in a 12-year-old with nasopharyngeal carcinoma (NPC) refractory to chemotherapy. Cytogenetics performed after death revealed a t(15;19) abnormality that was unusual for NPC.[4]

Despite its increased awareness, the actual incidence and molecular characteristics of NC remain unclear.[5] It is likely that NC is still underdiagnosed, given that recommended testing for a new diagnosis of non–small cell lung cancer includes only known actionable alterations such as *KRAS* and *EGFR* (by DNA NGS) or *ALK* and PD-L1 (by immunohistochemistry [IHC] or fluorescence in situ hybridization [FISH]) and recommended testing for head and neck cancers only involves HPV (detection by PCR or FISH or implied by p16 IHC) and PD-L1 testing.[6-8] Thus, rearrangements not picked up on standard testing are likely to remain undetected.

Case series have reported the characteristics and survival of patients with NC. In 2012, data from the NUT Carcinoma Registry described 63 patients with NC in the United States and Europe.[9] The median age was 16 years (range, 0.1-

CONTEXT

Key Objective
What are the molecular characteristics of nuclear protein in testis carcinomas (NCs)?

Knowledge Generated
Fewer intratumoral immune cells by RNA sequencing were observed in NC, which may be associated with the lack of benefit to immunotherapy. High MYC pathway activity in NC supports ongoing trials targeting MYC suppression.

Relevance
Future studies for NC should focus on improving the response to immunotherapy and targeting MYC.

78 years old), and most tumors originated in the lungs (56%) or head and neck (21%). Of these, 38 had *NUTM1*::*BRD4* fusion, three had *NUTM1*::*BRD3*, and 12 had uncharacterized *NUTM1* fusion partners. The median overall survival (OS) was 6.7 months.

NC lacks treatment options. Chemotherapy has limited efficacy. Xie et al reported outcomes from seven patients with primary lung NC.[10] The initial treatment included chemotherapy in five patients and surgery and radiotherapy in one patient each. Immunotherapy was used as a second-line or later therapy in five patients. The OS was 4.1 months (range, 1.5-26.7 months).[10] The updated NUT Carcinoma Registry found that ifosfamide-based chemotherapy had an increased 12-month disease-free survival (DFS) for patients with nonmetastatic disease, with a 12-month DFS of 59% versus 37% for platinum-based chemotherapy.[11] However, there was no significant effect on the 3-year OS, which was 19% in all patients.

Currently, there is no effective targeted treatment for NC. Trials for NC focus on inhibiting bromodomain extraterminal (BET) proteins (eg, BRD4), but the development of these drugs has been limited because of dose-limiting toxicities. In particular, severe thrombocytopenia and neutropenia were side effects in 40% of the patients.[12]

Because of the limited data on the molecular characteristics of NC, we sought to molecularly characterize a series of NCs from a large database of DNA and RNA sequencing in hopes of ultimately guiding the development of effective treatments for NC.

METHODS

Patient Samples

An institutional review board–exempt, retrospective assessment of a deidentified molecular profiling database was surveyed for all formalin-fixed, paraffin-embedded (FFPE) solid tumor samples that underwent RNA-based tumor profiling at a Clinical Laboratory Improvement Amendments–certified laboratory (Caris Life Sciences, Phoenix, AZ) for comprehensive genomic profiling; all unique cases with successful fusion testing by targeted RNA sequencing were considered, and any harboring NUTM1 fusion was subjected to centralized pathologic review by a board-certified pathologist (M.G.E.) to confirm the diagnosis of NC.

Fusion Detection

NUTM1 fusions were detected using either the ArcherDx fusion assay (Archer FusionPlex Solid Tumor panel, Integrated DNA Technologies, Coralville, IA) or the Illumina NovaSeq platform (Illumina, Inc, San Diego, CA) with the use of the Agilent SureSelect Human All Exon V7 bait panel (Agilent Technologies, Santa Clara, CA). For the ArcherDx fusion assay, FFPE tumor samples were microdissected to enrich the sample to ≥20% tumor nuclei and mRNA was isolated and reverse transcribed into cDNA. Unidirectional gene-specific primers were used to enrich for target regions, followed by gene sequencing by synthesis (Illumina MiSeq platform). For fusion detection using the Illumina NovaSeq platform, FFPE specimens underwent pathology review to diagnose percent tumor content and tumor size; a minimum of 10% of tumor content in the area for microdissection was required to enable enrichment and extraction of tumor-specific RNA. The RNA FFPE tissue extraction kit (Qiagen LLC, Germantown, MD) was used for extraction, and the RNA quality and quantity were determined using the TapeStation (Agilent Technologies).

DNA Sequencing

Genomic DNA isolated from FFPE tumor samples was analyzed using the NextSeq or NovaSeq 6000 platform (Illumina, Inc). A custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies) assessed using NextSeq. For the tumor sample sequenced on the NovaSeq 6000 platform, more than 700 clinically relevant genes at high coverage and high read depth were used, along with another panel designed to enrich for an additional >20,000 genes at lower depth. All variants were detected with >99% confidence on the basis of allele

frequency and amplicon coverage, with an average sequencing depth of coverage of >500 and an analytic sensitivity of 5%. Genetic variants identified were interpreted by board-certified molecular geneticists as previously described.[13] Gene copy number was determined using a custom pipeline on the basis of CNVkit.[14] Using whole-exome sequencing by separate DNA extraction, ≥six copies were consistent with amplification. Tumor mutational burden was measured by counting all nonsynonymous missense, nonsense, in-frame insertion/deletion, and frameshift mutations found per tumor that had not been previously described as germline alterations in dbSNP151 and Genome Aggregation Database databases or benign variants identified by Caris geneticists. High TMB (TMB-H) was defined by a cutoff of ≥10 mutation/megabase (mut/Mb), on the basis of KEYNOTE-158 trial, which determined the cutoff of TMB of ≥10 mut/Mb.[15]

Whole-Transcriptomic Sequencing

FFPE tissue sections mounted on glass slides underwent staining with nuclear fast red. Regions that contained a minimum of 10% tumor content were delineated for manual microdissection. Whole-transcriptome sequencing (WTS) was executed using the Illumina NovaSeq platform (Illumina, Inc) along with the Agilent SureSelect Human All Exon V7 bait panel (Agilent Technologies), with the resulting data reported transcripts per million.

Gene Expression Profiling and Signatures

Gene Set Enrichment Analysis (GSEA) was performed using the WTS data and the Hallmark gene set collection from the Human Molecular Signatures Database.[16,17] Immune cell fraction was calculated using the quanTIseq pipeline, which used deconvolution of bulk transcriptomic data.[18] Transcriptomic signatures of NC were compared with those of the squamous cell carcinoma of head and neck, and lungs.

IHC

IHC was conducted on complete sections of FFPE tissues mounted on glass slides. PD-L1 expression was determined using the primary antibody SP142 (Abcam, Cambridge, United Kingdom), with a positive threshold of ≥2+ staining intensity and ≥5% of tumor cells stained. In cases where the lungs were indicated as the primary tumor site, primary antibody 22c3 pharmDx (Dako, Agilent Technologies, Santa Clara, CA) was used.

Real-World OS Data

Real-world OS information was obtained from insurance claims data and calculated from either time of biopsy to last contact. Hazard ratio (HR) was calculated using the Cox proportional hazard models, and *P* values were calculated using the log-rank test with significance determined as *P* value of <.05.

RESULTS

Patient Characteristics and Co-Occurrence of Other Genomic Events in NC

On pathologist review of all 160 tissue samples harboring *NUTM1* fusions, cases of porocarcinoma and *NUTM1*-rearranged sarcoma were excluded, and the remaining 54 were diagnosed with NC. A total of 54 cases were included in the final analysis: nearly half had *NUTM1*::*BRD4* fusions, with the remainder having *NUTM1*::*NSD3* and *NUTM1*::*BRD3* fusions (Fig 1; Table 1). The median age of the patients was 55 years (range, 23-77 years). The lung (59%) and head and neck (33%) were the primary tumor sites in most cases (Table 1). The most common fusion oncogene at these primary sites was *NUTM1*::*BRD4*, except that all three cases of thyroid origin had *NUTM1*::*NSD3* fusions.

Altered genes were primarily involved in epigenetic/histone modification pathways (7 of 50, 14%) or cell cycle regulation pathways (6 of 51, 12%), such as *KMT2C/D* (3 of 45, 7%), *KDM6A* (2 of 48, 4%), and *TP53* (2 of 50, 4%; Fig 2). Only two of 49 samples exhibited copy number amplifications (4%), found in *FGFR1/3* and *NUTM1*. *NUTM1* fusions were not significantly associated with any specific genomic mutation. TMB-H and loss of heterozygosity (LOH) were not observed in NC—0% had TMB ≥10 mut/Mb, and 0% had gLOH (defined as LOH in 16% of the segments analyzed). In comparison with non-NC head and neck squamous cell carcinoma (HNSCC) and lung squamous cell carcinoma (LUSC), NC had significantly lower rates of gLOH and TMB-high ($P < .0001$).

Assessment of the Tumor Microenvironment and Pathways of NC in Comparison With Other Squamous Cancers

Tumor PD-L1 expression was identified in 26% of the cases (12 of 47; three [SP142 IHC]; nine [22C3 pharmDx IHC]). To assess differences between NC and non-NC squamous cell carcinomas, the tumor microenvironment and gene expression of NC were compared with those non-NC HNSCC and non-NC LUSC. To ensure that the sample sizes of HNSCC and LUSC do not influence the interpretation of the result, a random selection of samples five times the size of NC samples was selected for both non-NC HNSCC (n = 270) and non-NC LUSC (n = 270). The quantity of immune cell population in the tumor microenvironment was inferred using quanTIseq (Fig 3A). Macrophages (M1, M2), neutrophils, T cells ($CD4^+$ and $CD8^+$), and T regulatory cells were significantly lower in NC compared with non-NC HNSCC and non-NC LUSC (all *P* values were $P < .001$). On the other hand, B cells and monocytes were significantly increased in NC ($P < .001$, respectively; Fig 3A; Appendix Table A1). When comparing the tumor microenvironment on the basis of the primary tumor site of NC, there was no difference observed between NC from lung primary (n = 29) and those from head and neck primary (n = 16; Fig 3B).

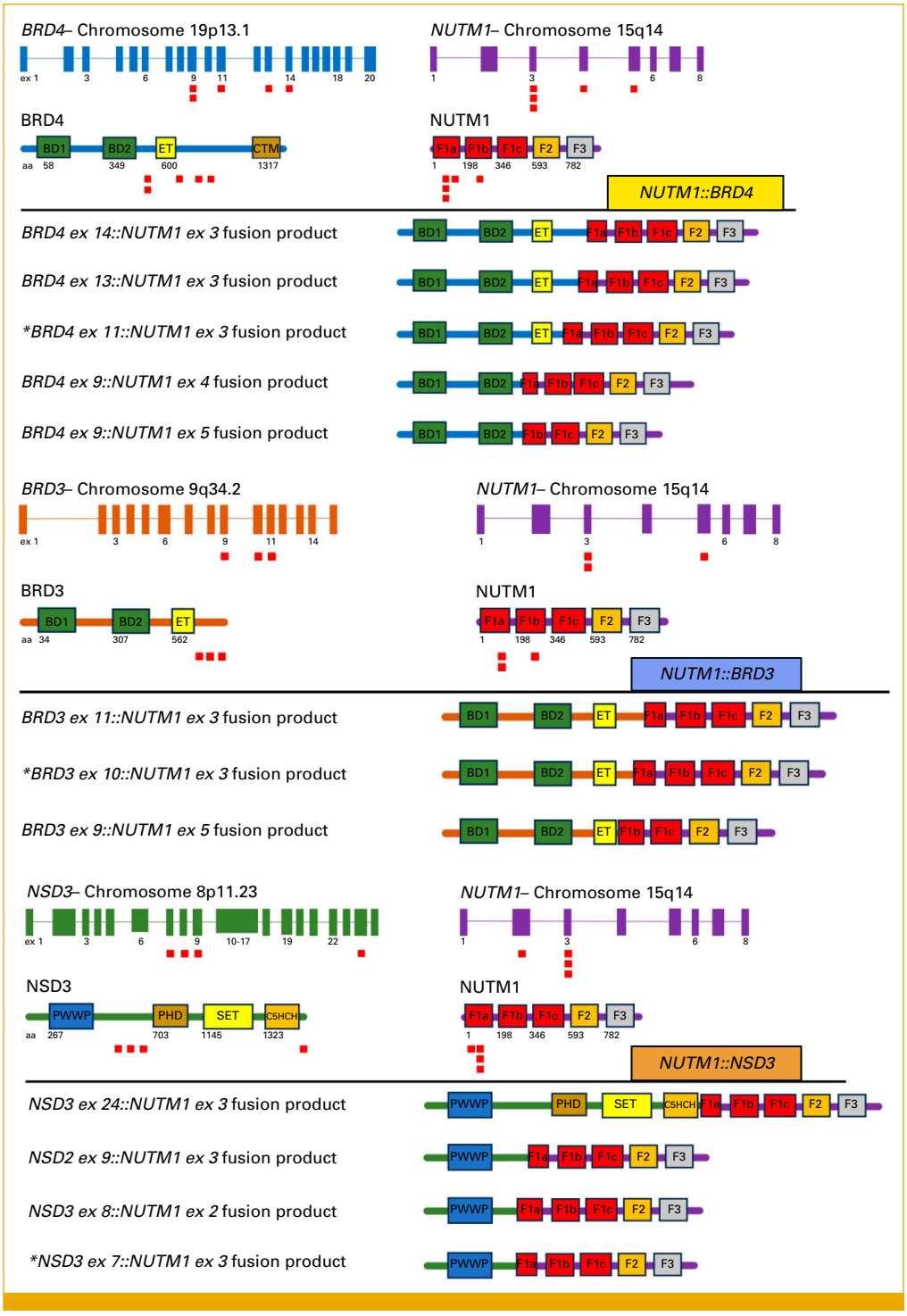

FIG 1. Detected NUTM1 fusions involving BRD4, BRD3, and NSD3 (WHSC1L1). The genes are illustrated at the genomic and protein transcript level with depiction of the various fusion products, including pertinent subdomains. Red boxes correspond to breakpoints. Asterisks represent the most frequently identified fusions per partner gene. aa, amino acid; ex, exon.

Similarly, immune-related gene expression (eg, *CTLA4*, *IFN-γ*, and *CD86*) in NC was lower compared with that in non-NC HNSCC (HNSCC, n = 270), as well as non-NC LUSC (n = 270). All the immune-related genes except for IDO1 had significantly higher fold change in non-NC HNSCC and non-NC LUSC relative to NC ($P < .05$; Fig 3C; Appendix Table A1).

GSEA pathway analysis demonstrated that the MYC targets v2 pathway was enriched in NC although this was not significant when compared with non-NC HNSCC or non-NC LUSC by GSEA (Appendix Fig A1). Interestingly, MYC targets v2 was significantly ($P < .05$) enriched in NC with a lung primary site compared with NC arising from the head and

TABLE 1. Patient Characteristics by *NUTM1* Gene Fusion Partner

Characteristics	Overall Cohorts (N = 54)	*BRD4* (n = 24)	*NSD3* (n = 22)	*BRD3* (n = 8)	*P*
Age at diagnosis, years					
Age, median (range)	55 (23-77)	50 (23-72)	57 (26-76)	62 (35-77)	.15
Sex, No. (%)					
Male	31 (57)	13 (54)	12 (55)	6 (75)	.61
Female	23 (43)	11 (46)	10 (45)	2 (25)	
Primary tumor sites, No. (%)					
Lung	29 (59)	12 (55)	11 (55)	6 (86)	—
Head and neck	16 (33)	9 (41)	6 (30)	1 (14)	
Thyroid	3 (6)	0	3 (15)	0	
Genitourinary	1 (2)	1 (4)	0	0	
Unknown	5	2	2	1	

neck (Appendix Figs A2A and A2B). Similarly, there was numerically higher expression of selected MYC-associated genes (Appendix Fig A2C) in NC with lung primary compared with NC with HN primary (median—MYC: 4.86 *v* 4.45; *P* = .689; PA2G4: 3.80 *v* 3.18; *P* = .022). There were no significant pathways enriched associated with specific fusion partners of *NUTM1*.

Treatment Profile and Prevalence of NC

The outcome data were limited (n = 32); however, there was a numeric benefit to treatment with platinum-based chemotherapy (cisplatin, carboplatin and oxaliplatin) versus no chemotherapy (OS no chemo *v* chemo: 6.9 months *v* 11.5 months; HR, 1.39 [95% CI, 0.57 to 3.3];

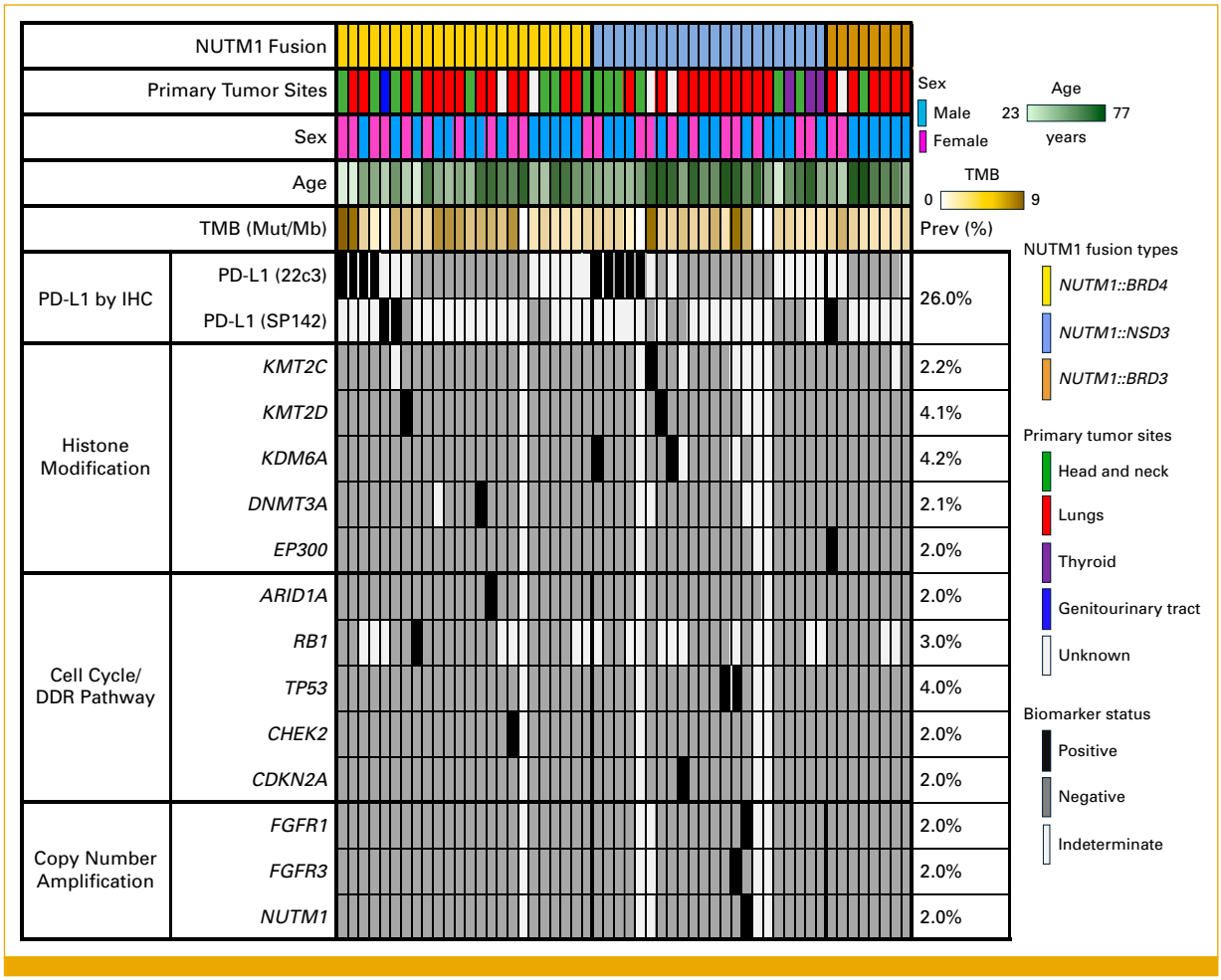

FIG 2. Oncoprint diagram with baseline characteristics and genomic coalterations, categorized by NUTM1 fusion partner. DDR, DNA damage response; IHC, immunohistochemistry; mut/Mb, mutation/megabase; TMB, tumor mutational burden.

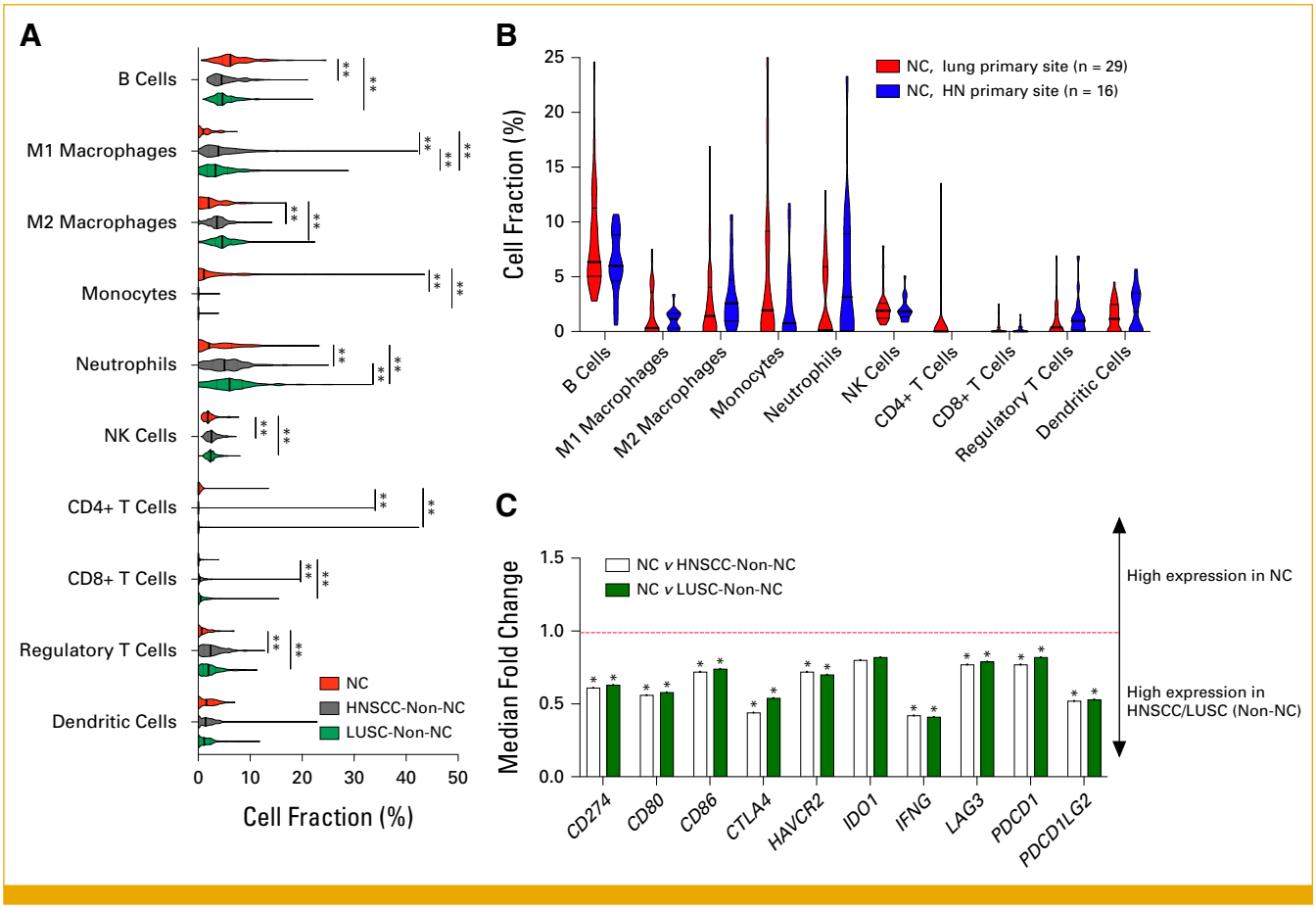

FIG 3. RNA expression of the NC cohort. (A) Differences in inferred immune cell populations in individual tumor samples by RNA sequencing in NC compared with HNSCC (nonNC) and LUSC (nonNC). Cell fraction represents % of all immune cells. (B) Differences in inferred immune cell populations in NC on the basis of primary tumor sites. (C) Fold change of immune-related genes in NC relative to HNSCC (non-NC) and LUSC (non-NC), with significance shown on each bar. HN, head and neck; HNSCC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; NC, nuclear protein in testis carcinoma. *P < .05; **P < .0001.

P = .46; Fig 4). As an exploratory analysis, KM analysis was used to compare the 17 patients who received chemo-immunotherapy (chemo-IO) versus chemotherapy alone. The median OS was similar in chemo-IO (10.29 months) and chemo-only (11.48 months); HR, 1.49 (95% CI, 039 to 5.5); P = .56 (Appendix Fig A3). To demonstrate if NUTM1 fusion partners were prognostic predictors, an exploratory KM analysis was considered between the NUTM1 fusion partners. The result showed no differences between the OS from biopsy to last contact (HR, approximately 1; P > .05; Appendix Fig A4).

Furthermore, the prevalence of NC was assessed in comparison with that of LUSC and HNSC. Of the 17,156 squamous cell cancers (SCCs) of the head, neck, and lungs accessed from the Caris database (accessed: September 26, 2023), 0.13% had NC-associated fusions (Fig 5; Appendix Fig A3). Prevalence decreased with age, with an incidence rate over 10× higher in patients younger than 50 years than in those older than 70 years.

DISCUSSION

In summary, to our knowledge, this is the largest series of NCs profiled broadly at both DNA and RNA levels. While NC is driven by *NUTM1* fusion oncoproteins, co-occurring mutations in epigenetic or cell cycle pathways were observed in 26% of cases and may play a role in the pathophysiology of NC. There were no significant differences between the fusion partner and co-mutations. RNA transcript analysis suggested increased *MYC* pathway activity in NC compared with that in non-NC HNSCC and non-NC LUSC. NC incidence is 10× higher in patients with HNSCC and LUSC younger than 50 years and older than70 years.

Characterization of the NC tumor microenvironment using RNA sequencing revealed significantly lower immune cell infiltration compared with non-NC HNSCC and non-NC LUSC. The lower proportions of $CD4^+$, $CD8^+$, and regulatory T cells were consistent with an immunosuppressive environment within the tumors. Notably, this is consistent

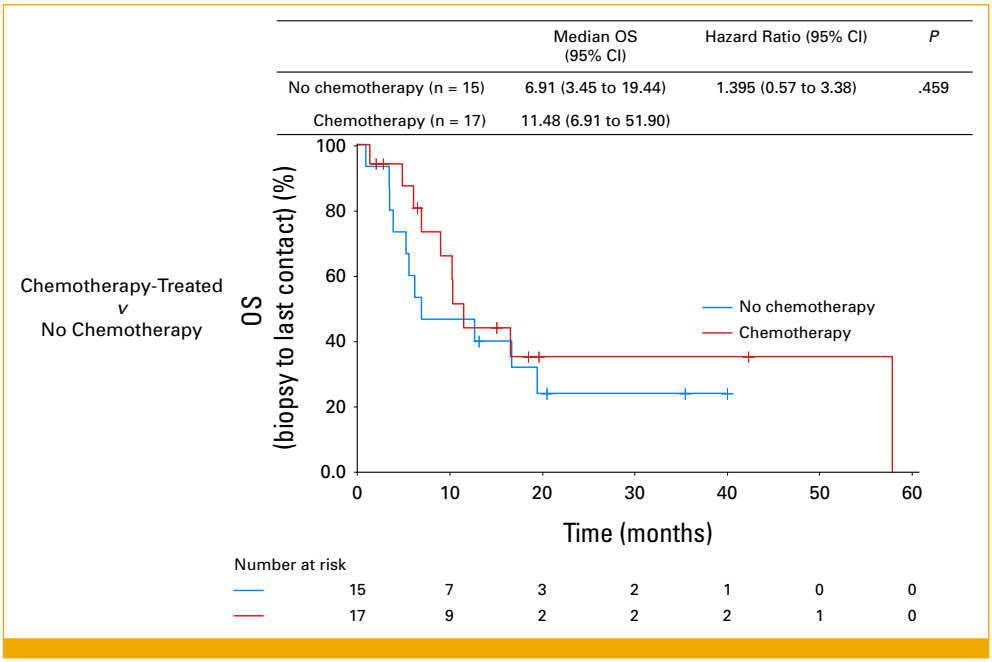

FIG 4. OS in chemotherapy-treated versus no chemotherapy–treated NC. NC, nuclear protein in testis carcinoma; OS, overall survival.

with a recent review by French et al.[19] The microdissection of tumors in this cohort was performed to exclude the periphery to maximize tumor cell sequencing, so the microenvironment characterized here is primarily intratumoral. The limited intratumoral immune cells and lower PD-L1 positivity may be associated with the diminished benefit to immunotherapy among SCCs; however, given the small sample size, this requires further study.

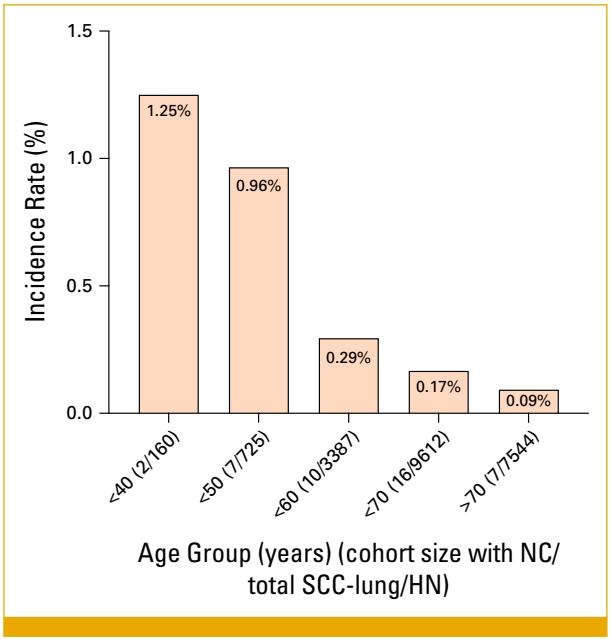

FIG 5. Rate of NC among squamous cell carcinomas of lungs and head and neck by age in those profiled by Caris. HN, head and neck; NC, nuclear protein in testis carcinoma; SCC, squamous cell cancer.

Our findings of increased MYC pathway transcripts in NC compared with non-NC HNSCC and non-NC LUSC in the GSEA analysis are consistent with the known pathophysiology of NC. MYC is a common driver of many solid tumors,[20] and NC is highly MYC addicted. Data from this study support that MYC suppressing agents may be effective in NC treatment. In addition, the low number of intratumoral immune cells may be partly explained by MYC overexpression. MYC downregulates MHC I expression and contributes to intratumoral immunosuppression.

The cohort, an expansion of previously reported data from the Caris' database,[21] to our knowledge, is the largest data set to estimate the incidence of NC in the United States. It should be noted that Caris does not contract with many pediatric hospitals, so the age distribution of this cohort is higher than the known age distribution of NC (median age of 55 years *v* 16 years and 34 years in previous reports). Because of this bias of not having many pediatric cases in the Caris database (whereas pediatric cases would likely include pediatric NC cases given a fast alternative test of NUT IHC being available in pediatric centers[22]), we estimated the incidence of NC using older individuals (age ≥40 years). In addition, we chose squamous cell carcinoma over poorly differentiated cancer as it is a consistently defined histology seen in NC. The US SEER database estimates approximately 159,000 cases of HNSCC and LUSC yearly in patients 40 years and older. Given a 0.12% incidence of NC in patients 40 years and older,

accounting for data that 2 of 3 patients with NC are younger than 40 years at diagnosis, and approximately 40% of NC samples have squamous cell carcinoma histology[11], the estimated US incidence of NC is approximately 1,400 cases per year (Fig 5; Appendix Fig A3).

NC is aggressive; therefore, early identification of this molecularly driven disease is critical.[23] Targeted BET inhibitors have shown on-target downregulation of NC target genes in cell line models and improved survival in murine models of NC.[24] Monotherapy trials (eg, GSK 525762, ClinicalTrials.gov identifier: NCT01587703; RO6870810, ClinicalTrials.gov identifier: NCT01987362; and birabresib MK-8268, ClinicalTrials.gov identifier: NCT02259114) have a response rate of 20%-30%, with limited durability. Molibresib or GSK 525762 demonstrated confirmed and unconfirmed responses in nine of 19 patients (21%) with NC in a phase I study.[25] RO6870810 demonstrated a 25% objective response rate and a 63% stable disease rate in NC, with a median PFS of 3.1 months.[26] Of 10 patients who received birabresib with NC, three (3 of 10, 30%) had partial response as a best response.[27]

Oral BET inhibitor combinations may overcome DLTs seen in monotherapy and lead to better durability in NC; recruiting studies include ZEN003694 + cisplatin and etoposide (ClinicalTrials.gov identifier: NCT050197160), ZEN003694 + CDK4/6 inhibitor abemaciclib (ClinicalTrials.gov identifier: NCT05372640), CDK9 inhibitor KB-0742 (ClinicalTrials.gov identifier: NCT04718675), dual BET and CBP/p300 inhibitor EP31670 (ClinicalTrials.gov identifier: NCT05488548), and BRD plus BET inhibitors of BMS-986158 and BMS-986378 (ClinicalTrials.gov identifier: NCT03936465). The rapid identification of NC cases is essential to match patients to promising ongoing clinical trials.

Given the incidence estimates of NC across age groups, testing for NC is performed more frequently for SCCs, especially in those younger than 40-50 years. Recognizing that NGS may be inaccessible for some, NUT IHC and FISH are diagnostic tools for NC and can greatly improve the rapid identification of this disease.[22]

The limitations of our study include the lack of pediatric patients because of the inability to control for broad DNA and RNA NGS. It is possible that some samples were treatment-naïve, and some samples were pretreated, so the genomic alterations and the tumor microenvironment might have been a result of treatment. Outcome data were limited by the sample size and were derived from insurance claims. Although the survival of chemotherapy-treated patients was better, it was confounded by individuals who were too ill for treatment. Given the relatively small overall sample size, we were unable to perform multivariable analysis with confidence in this study. Nevertheless, this study is one of the largest cohorts of patients with NC to date. In addition, to our knowledge, this is the largest study to characterize the incidence rate of NC in comparison with squamous cell carcinoma of the lungs and head and neck.

In conclusion, NC is associated with a low tumor mutational burden and a general lack of additional mutations in known cancer-associated genes. The association between epigenetic and cell cycle gene alterations should be functionally explored. NC is underdiagnosed, and testing for NC should be routine in those age 40-50 years and younger with squamous cell carcinoma of the lungs, head, and neck. The prognosis of NCs remains dismal, and future investigations should focus on targeting MYC overexpression, detecting new targets for antibody drug conjugates, and developing strategies to enhance the immune response.

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EQUAL CONTRIBUTION

G.K. and J.L. contributed equally to the manuscript and are considered cofirst authors.

PRIOR PRESENTATION

Preliminary report on this content (genomic landscape of NUT carcinoma) has been presented as a poster at the Association for Molecular Pathology Meeting, Salt Lake City, November 18, 2023.

DATA SHARING STATEMENT

All data generated or analyzed during this study are included in this manuscript and its supplementary files. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. The deidentified sequencing data are owned by Caris Life Sciences and cannot be publicly shared due to the data usage agreement. Qualified researchers can apply for access to these summarized data by contacting Caris Life Sciences and signing a data usage agreement. The authors will honor legitimate requests for data sharing to qualified researchers, upon request, as necessary for conducting methodologically sound research.

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Administrative support: Misako Nagasaka
Provision of study materials or patients: Ammar Sukari, David R. Braxton
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Data analysis and interpretation: Gianna Kroening, Jia Luo, Mark G. Evans, Tolulope Adeyelu, Sai-hong Ignatius Ou, Trisha M. Wise-Draper, Ammar Sukari, Asfar S. Azmi, David R. Braxton, Andrew Elliott, Chul Kim, Geoffrey Shapiro, Christopher French, Misako Nagasaka
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Consulting or Advisory Role: AstraZeneca, Astellas Pharma, Amgen
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Travel, Accommodations, Expenses: Erasca, Inc, Blueprint Medicines, Daiichi Sankyo

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Employment: Caris Life Sciences
Stock and Other Ownership Interests: Caris Life Sciences
Travel, Accommodations, Expenses: Caris Life Sciences

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Consulting or Advisory Role: Pfizer, Takeda, Janssen, Daiichi Sankyo, AnHeart Therapeutics
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Research Funding: Merck, AstraZeneca/MedImmune, Bristol Myers Squibb, GlaxoSmithKline, Caris Life Sciences, GlaxoSmithKline, Janssen Oncology
Travel, Accommodations, Expenses: Caris Life Sciences

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Honoraria: Merck KGaA
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Patents, Royalties, Other Intellectual Property: Patent number: 9,872,874 Title: Dosage regimen for sapacitabine and seliciclib Issue Date: 1/23/2018, Patent number 10,934,593 B2. Compositions and methods for predicting response and resistance to CDK4/6 inhibition

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Research Funding: Tempus
Travel, Accommodations, Expenses: Anheart Therapeutics

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APPENDIX

TABLE A1. Summary Statistics of the Tumor Microenvironment and Gene Expression Level of NC in Comparison With HNSCC and LUSC

Median Cell Fraction (%)	NC (n = 54)	HNSCC (n = 270)	LUSC (n = 270)	Pairwise *q*-Value (NC *v* HNSCC)	Pairwise *q*-Value (NC *v* LUSC)	Pairwise *q*-Value (HNSCC *v* LUSC)
B cells	6.189	4.500	4.669	0.000	0.000	0.638
M1 macrophages	0.950	3.881	3.324	0.000	0.000	0.005
M2 macrophages	2.033	3.614	4.627	0.001	0.000	0.000
Monocytes	1.096	0.000	0.000	0.000	0.000	0.185
Neutrophils	2.074	5.061	6.047	0.001	0.000	0.012
NK cells	1.866	2.553	2.415	0.000	0.000	0.145
CD4 T cells	0.000	0.000	0.000	0.003	0.001	0.545
CD8 T cells	0.000	0.310	0.448	0.001	0.000	0.102
T regulatory cells	0.688	2.379	1.970	0.000	0.000	0.064
Dendritic cells	1.585	1.429	1.170	0.878	0.508	0.241

Median Expression (TPM)	Median (NC)	Median (HNSCC)	Median (LUSC)	Pairwise *q*-Value (NC *v* HNSCC)	Pairwise *q*-Value (NC *v* LUSC)	Pairwise *q*-Value (HNSCC *v* LUSC)
CD86	2.412	3.341	3.248	0.000	0.000	0.687
CD80	1.539	2.742	2.674	0.000	0.000	0.135
LAG3	0.829	1.074	1.056	0.032	0.009	0.729
HAVCR2	2.898	3.997	4.137	0.000	0.000	0.444
CD274	2.000	3.278	3.194	0.000	0.000	0.851
IDO1	1.972	2.474	2.393	0.141	0.162	0.843
PDCD1LG2	0.742	1.426	1.388	0.000	0.000	0.865
PDCD1	0.518	0.670	0.630	0.043	0.135	0.444
IFNG	0.284	0.680	0.690	0.000	0.000	0.869
CTLA4	0.868	1.975	1.601	0.000	0.000	0.000

Abbreviations: HNSCC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; NC, nuclear protein in testis carcinoma; TPM, transcripts per million.

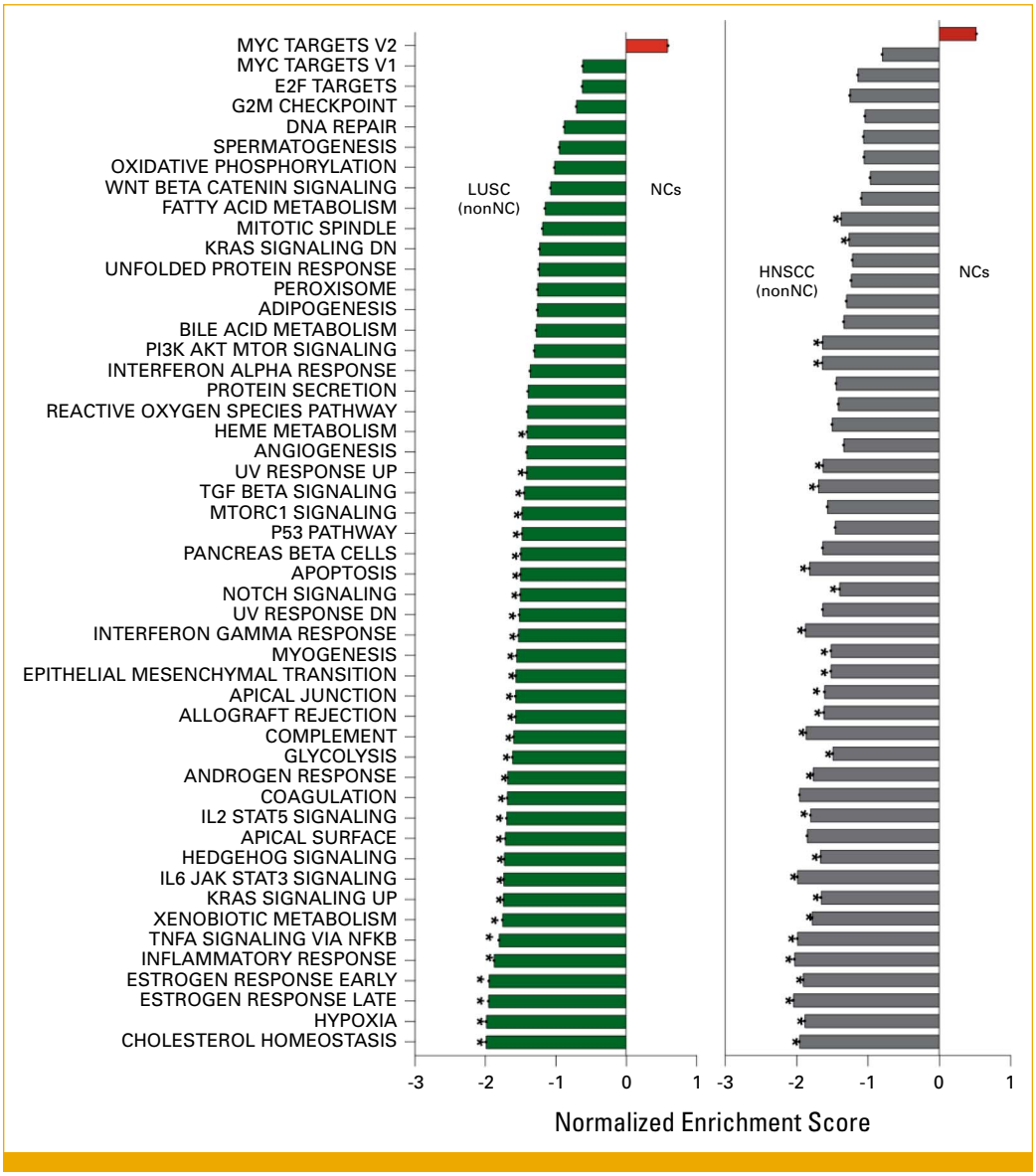

FIG A1. Pathway analysis comparing NC versus HNSCC (non-NC) versus LUSC (non-NC) sorted by NUT versus LUSC (non-NC). Shading represents the normalized enrichment score from GSEA between the cohorts, whereas pathways that are statistically significant are marked with asterisks. GSEA, Gene Set Enrichment Analysis; HNSCC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; NC, nuclear protein in testis carcinoma; NUT, nuclear protein in testis.

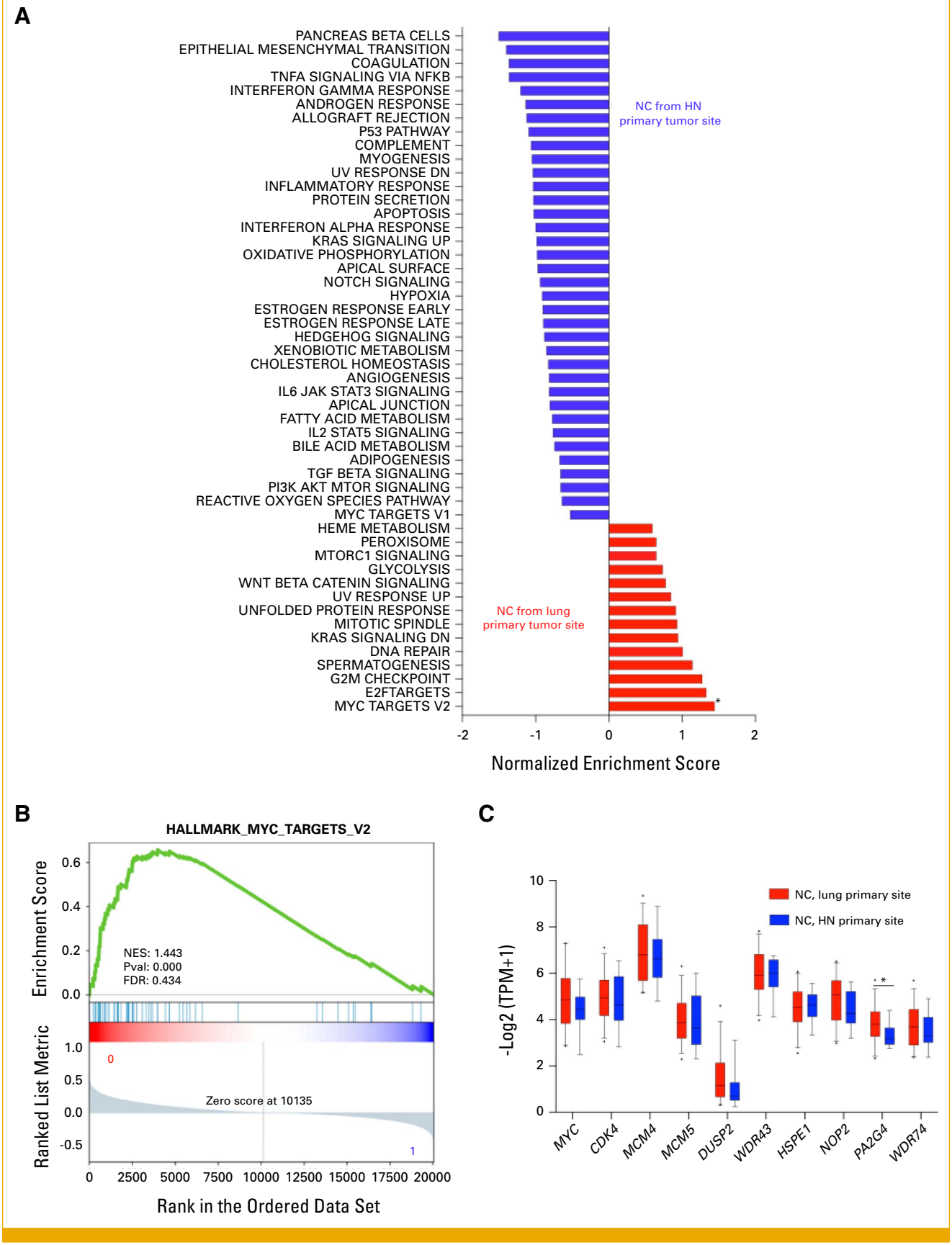

FIG A2. Gene Set Enrichment Analysis results of NC with primary lung versus primary head and neck cancer. (A) Bar plot showing the distribution of the normalized enrichment score between cohorts. (B) GSEA plot of MYC target V2 (continued on following page)

FIG A2. (Continued). between cohorts. (C). Distribution of gene expression levels between cohorts. Shading represents the normalized enrichment score from GSEA between cohorts, whereas the *P* value represents the significance of enrichment. GSEA, Gene Set Enrichment Analysis; HN, head and neck; NC, nuclear protein in testis carcinoma; NES, normalized enrichment score; TPM, transcripts per million.

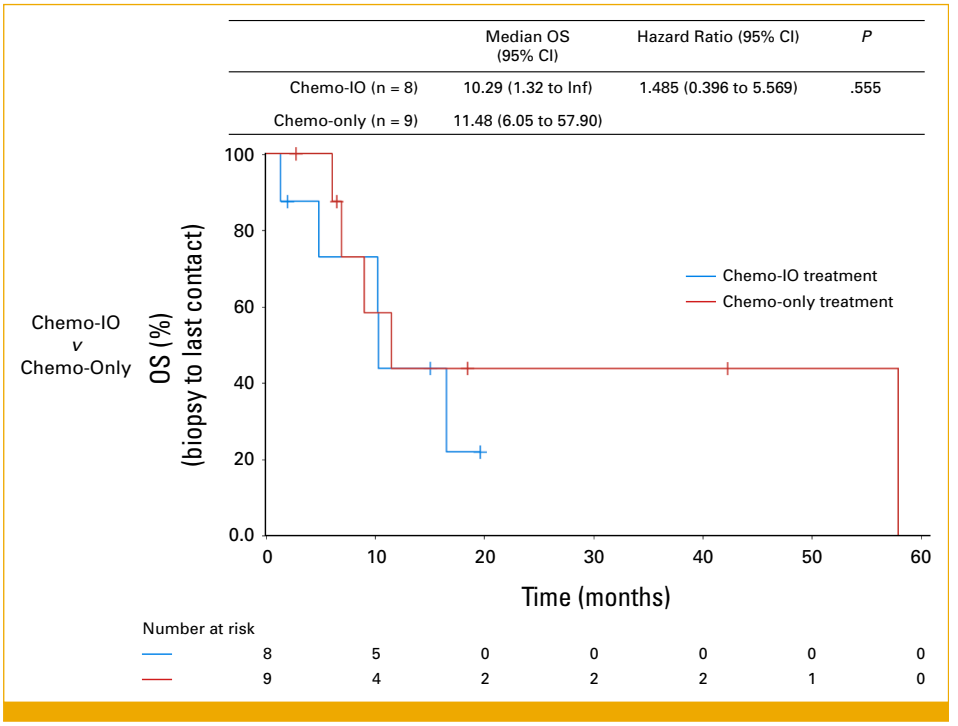

FIG A3. OS in chemo-treated versus chemo-IO–treated NC. chemo-IO, chemoimmunotherapy; Inf, infinite; NC, nuclear protein in testis carcinoma; OS, overall survival.

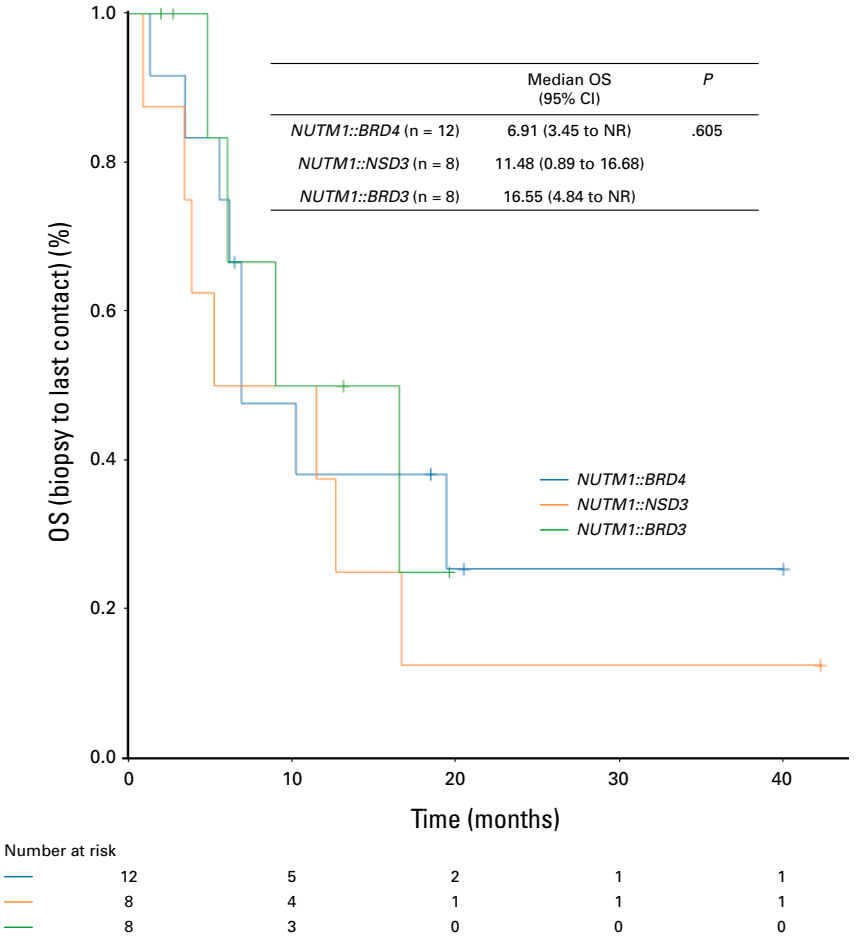

FIG A4. OS compared among different NUTM1 partners from biopsy to last contact. NR, survival not reached; OS, overall survival.

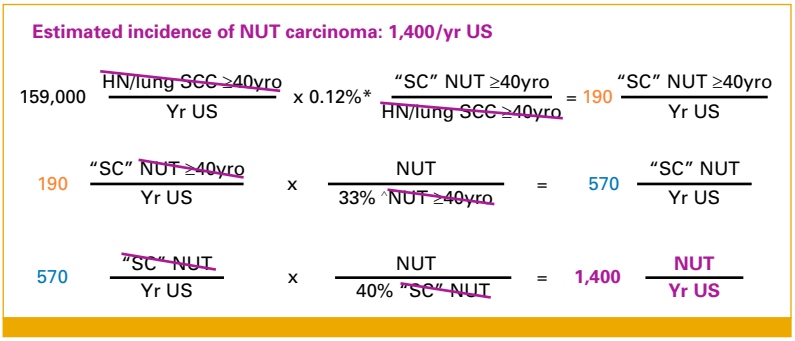

FIG A5. Incidence rate calculation for NUT carcinoma. HN, head and neck; NC, nuclear protein in testis carcinoma; NUT, nuclear protein in testis; SC, squamous cell; SCC, squamous cell cancer. [a]Caris has no pediatric practice contracts. [b]Approximately 33% of NC is observed in patients older than 40 years, and approximately 40% of NC is squamous per Luo the study by et al.[11]