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Molecular Profiling Reveals Limited Targetable Biomarkers in Neuroendocrine Carcinoma of the Cervix

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Abstract: Neuroendocrine carcinoma of the cervix (NEC) is a rare and highly aggressive cervical malignancy. Given that no targeted therapy has been approved specifically to NEC, we investigated the presence of novel, potentially targetable biomarkers in a large cohort of NEC. Sixty-two NEC were molecularly profiled for biomarkers of targeted therapies including antibody-drug conjugates [delta-like canonical notch ligand 3 (DLL3), a trophoblast cell surface antigen 2 (TROP-2), and folate receptor 1 (FOLR1)], *NTRK1-3* gene fusions, and immune checkpoint inhibitors [programmed death-ligand 1 (PD-L1), tumor mutational burden, and microsatellite instability] using immunohistochemistry and DNA/RNA next-generation sequencing assays. A cohort of squamous cell carcinomas of the cervix (n = 599) was used for comparison for immune-oncology biomarkers. DLL3 expression was observed in 81% of the cases. DLL3 expression was inversely correlated with commonly observed pathogenic mutations in *PIK3CA* (17%) ($P = 0.018$) and *PTEN* (10%) ($P = 0.006$). Other more frequently seen pathogenic mutations (*TP53* 17%, *KRAS* 11%, and *CTNNB1* 5%) were not associated with DLL3 expression. TROP-2 expression was detected in only 1 case and no case expressed FOLR1. Although NTRK protein expression was observed in 21% of the cases, none of these had *an NTRK* gene fusion. PD-L1 expression (10%) and high tumor mutational burden (3%) were significantly less frequent in NEC compared with the squamous cell carcinoma cohort (79% and 11%, respectively). None of the NEC exhibited high microsatellite instability status. Despite frequent DLL3 expression in NEC, a potential therapeutic benefit of DLL3-targeted drugs remains uncertain given the recent failure of the Rova-T therapeutic trial in small cell lung carcinomas. Small cohorts of NEC enriched in PIK3CA/PTEN/AKT and programmed cell death protein 1/PD-L1 alterations indicate therapeutic roles for their respective inhibitors.

Key Words: cervix, neuroendocrine carcinoma, molecular profiling, sequencing, targeted therapy

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Neuroendocrine carcinoma of the cervix (NEC) is a rare primary cervical cancer encompassing ~1% of all cervical malignancies.[1–3] These cancers tend to have a high propensity to spread both locally and distally, and affected patients usually present with advanced disease (eg, liver and lung metastases) at initial presentation.[4,5] The prognosis is very poor with a mean overall survival of ~40 months.[1–3] Similar to other subtypes of cervical carcinomas, NECs are also associated with high-risk human papillomavirus (HPV) infections (HPV16 and HPV18 as the most common).[1,6]

The current therapeutic approaches to NEC patients mainly derive from NECs of the lung.[1] These usually include radical hysterectomy followed by adjuvant chemotherapy (cisplatin/carboplatin and etoposide) or concurrent chemotherapy and radiation therapy for early disease; concurrent chemotherapy and radiation therapy that may be preceded by neoadjuvant chemotherapy and followed by adjuvant chemotherapy is used for locally advanced NEC, while palliative chemotherapy is the modality treatment for patients with metastatic NEC.[1,3]

Limited data are available regarding the targetable molecular features of NEC.[7–11] Given that no targeted therapy has been approved yet for NEC, we explored novel targetable biomarkers in a large cohort of NEC.

MATERIALS AND METHODS

Samples

Sixty-two NEC of the cervix were profiled for biomarkers of targeted therapies at Caris Life Sciences (Phoenix, AZ). All samples were submitted by referring pathologists and oncologists for molecular profiling purposes. >90% of the samples were provided from the pathology laboratories from

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the United States. A cohort of squamous cell carcinomas (SCCs) of the cervix (n = 599) was used for comparison for immune-oncology biomarkers.

Before molecular testing, each NEC case underwent confirmation of the histologic diagnosis and a review of the diagnostic immunohistochemical (IHC) workup [eg, neuroendocrine markers (synaptophysin, chromogranin-A, CD56), cytokeratins (AE1-AE3, Cam5.2, CK5/6, CK7, and CK20), p16, p40, TTF-1] performed at the referring pathology laboratories. All reports were deidentified and remnant NEC samples provided by the referring laboratories. Given that the remnant tissues from previous samplings with no associated identifiers were used, this research was compliant with 45 CFR 46.101(b). Therefore, the present study was deemed exempt from Institutional Review Board approval and consent requirements were waived. The study was conducted in compliance with the guidelines for human studies provided by the World Medical Association Declaration of Helsinki.

All molecular assays were performed at a CLIA/CAP/ISO15189/NYSDOH certified clinical laboratory (Caris Life Sciences).

IHC

IHC assays included stains against the following biomarkers: delta-like canonical notch ligand 3 (DLL3), a trophoblast cell surface antigen 2 (TROP-2), folate receptor 1 (FOLR1), neurotrophic receptor tyrosine kinase 1-3 (NTRK), and programmed death-ligand 1 (PD-L1). We summarized in Table 1 the information regarding the antibodies, thresholds for positivity, and subcellular localization of each biomarker.[12,13] All IHC assays were run with both positive and negative controls.

HPV Detection [In Situ Hybridization (ISH)]

For HPV assessment, we used a cocktail of 3 mRNA probes for high-risk HPVs 16, 18, and 33. The HPV-ISH assay was performed following the manufacturer's instructions with the BenchMark automated slide staining system (Ventana Medical Systems).

Next-generation Sequencing (NGS)

The NEC samples were profiled using massively parallel sequencing (NGS) of exons from 592 genes (SureSelect XT; Agilent, Santa Clara, CA, and the NextSeq instrument; Illumina, San Diego, CA).[13,14] A full gene panel is available at: www.carismolecularintelligence.com/wp-content/uploads/2017/03/TN0276-v5_Profile-Menu-Brochure.pdf.

The tumor mutational burden (TMB) was assessed by calculating the number of nonsynonymous missense mutations, excluding common germline variants, per 1 Mbp of DNA. TMB was considered high if ≥ 17 mutations/Mbp were detected. The estimated threshold was based on a cohort of 599 cases of SCC of the cervix that was profiled at Caris Life Sciences. We applied the 80th percentile cutoff value for TMB assessment as suggested by Samstein et al.[15]

Microsatellite instability (MSI) was calculated from the NGS data by direct analysis of short tandem repeat tracts in the target regions of sequenced genes. The count only included alterations that resulted in increases or decreases in the number of repeats; high MSI was defined as ≥ 46 altered microsatellite loci. This threshold was established by comparing NGS with the polymerase chain reaction–based microsatellite fragments analysis results from ~2100 samples.[16] MSI status was compared with that from a cohort of SCC of the cervix (n = 599).

Copy number alterations were assessed by comparing the depth of detected NGS sequence reads to reads from a diploid control. Genes having ≥ 6 copies were considered amplified.[13,14,17–19]

The ArcherDx FusionPlex Assay (ArcherDX, Boulder, CO) was used for the gene fusion assessment. The gene fusions panel (n = 54) is available at: www.carismolecularintelligence.com/wp-content/uploads/2017/03/TN0276-v14_Profile-Menu.pdf.

Statistical Methods

The χ^2 and Fisher exact tests were used to analyze the relationships between the examined variables. *P*-values < 0.05 were considered significant. IBM SPSS Statistics (version 25) was used for statistical analysis (licensed to Qatar University).

RESULTS

Clinicopathologic Characteristics of the Cohort

The study group included 36 primary and 26 metastatic NEC of the cervix. Metastatic sites of cervical NEC included pelvis (and pelvic lymph nodes) (n = 6), liver (n = 5), uterus (n = 2), ovary (n = 1), vagina (n = 1), lung (n = 1), brain (n = 1),

TABLE 1. Overview of the Antibodies That Were Used in the Study

Antibody	Clone (Manufacturer)	Threshold for Positivity	Subcellular Localization
Delta-like canonical notch ligand 3 (DLL3)	SP347 clone (Ventana)	Any positivity ≥ 50% (high expression)	Membranous/cytoplasmic
Trophoblast antigen 2 (TROP-2)	Anti-human Trop-2 (R&D Systems)	≥ 10%, 2+ intensity > 50% (high expression)	Membranous
Folate receptor 1 (FOLR1)	Clone 26B3.F2 (Biocare Medical)	*H*-score ≥ 1 (≥ 20 for high)	Membranous
Tropomyosin receptor tyrosine kinase (1-3) (Pan-TRK)	Clone EPR17341 (Abcam)	≥ 1% of tumor cells	Membranous/cytoplasmic and nuclear
Programmed death ligand 1 (PD-L1)	22C3 pharmDx Kit (Dako Agilent)	CPS ≥ 1*	Membranous/cytoplasmic

*CPS = the number of PD-L1+ cells (cancer cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100; PD-L1 positivity is defined as CPS ≥ 1.

CPS indicates Combined Positive Score.

TABLE 2. Comparison of Novel Predictive Biomarkers With Therapeutic Implications Between NECs of the Cervix and Other Common Cancers

Biomarker	Function	Mechanism of Action	Targeted Drug	Common Cancers	Diagnostic Assay	Status in NEC
DLL3	Notch ligand	Overexpression	Rovalpituzumab tesirine	SCLC	IHC	38+/47 (80%)
pNTRK	Nerve development and growth (activation by neurotrophins)	Protein overexpression due to gene fusion	TRK inhibitors (eg, entrectinib)	Pediatric sarcomas, thyroid, MASC, gliomas	IHC and Archer Fusion assay (NGS)	10+/47 (21%) positive by IHC *No TRK1-3 gene fusions*
FOLR1	Folate antimetabolites (eg, pemetrexed-therapy)	Overexpression	Imaging probes, drug conjugates, farletuzumab	NSCLC, breast, ovarian, CRC	IHC	0/47 (0%)
TROP-2	Transmembrane protein (intracellular calcium transducer)	Overexpression	Trop-2-targeted antibody-drug conjugates	Cervix (SCC), breast, CRC, esophagus, gliomas	IHC	1+/47 (2%)

CRC indicates colorectal carcinoma; IHC, immunohistochemistry; MASC, mammary analog secretory carcinoma; NEC, neuroendocrine carcinoma; NGS, next-generation sequencing; NSCLC, non–small cell lung cancer; SCC, squamous cell carcinoma; SCLC, small cell lung cancer.

peritoneum (n = 1), scapular region (n = 1), trunk (n = 1), and other distant lymph nodes (retroperitoneal, mediastinal, and supraclavicular) (n = 6). All cases were high-grade carcinomas. All cases were histopathologically composed of a monotonous population of small cells with hyperchromatic nuclei, frequent mitosis, nuclear molding, and scanty cytoplasm. Although the 2014 World Health Organization (WHO) classification[20] does not require the presence of any neuroendocrine markers, in all cases at least 1 neuroendocrine marker was positive to support the NEC diagnosis.

The mean patient's age with NEC was 43.6 years (range, 24 to 82 y). HPV results were available for 47 cases: 20 cases (43%) were positive by HPV-ISH, while the remaining 27 cases (57%) were negative. The HPV results were not associated with patients' age ($P = 0.38$) and tumor site (primary vs. metastatic NEC) ($P = 1.0$).

Antibody-drug Conjugate (ADC) Targets in NEC

The results of ADC biomarkers are summarized in Table 2.

DLL3 expression was observed in 81% of the cases with 49% of cases expressing diffusely (≥ 50% of positive cancer cells) DLL3 protein (Fig. 1). Metastatic NEC tended to have higher DLL3 expression compared with the primary NECs ($P = 0.08$). DLL3 expression did not differ significantly in regards to HPV status but was inversely correlated with commonly observed pathogenic mutations in *PIK3CA* (17%) ($P = 0.018$) and *PTEN* (10%) ($P = 0.006$). Other more frequently seen pathogenic mutations (*TP53* 17%, *KRAS* 11%, and *CTNNB1* 5%) were not associated with DLL3 expression.

TROP-2 expression was positive in 1 case, while FOLR1 showed no expression in any of the tested cases.

NTRK Status in NEC

NTRK expression using pan-TRK antibody was positive in 10+/47 (21%) cases (Seven primary and 3 metastatic NEC). However, no *NTRK1-3* gene fusions were detected in any case including the 10 cases positive for NTRK by IHC (Table 2, Fig. 2).

Genomic Profile of NEC

Pathogenic mutations were detected in 23 genes. The most commonly mutated genes included *PIK3CA* (11/62),

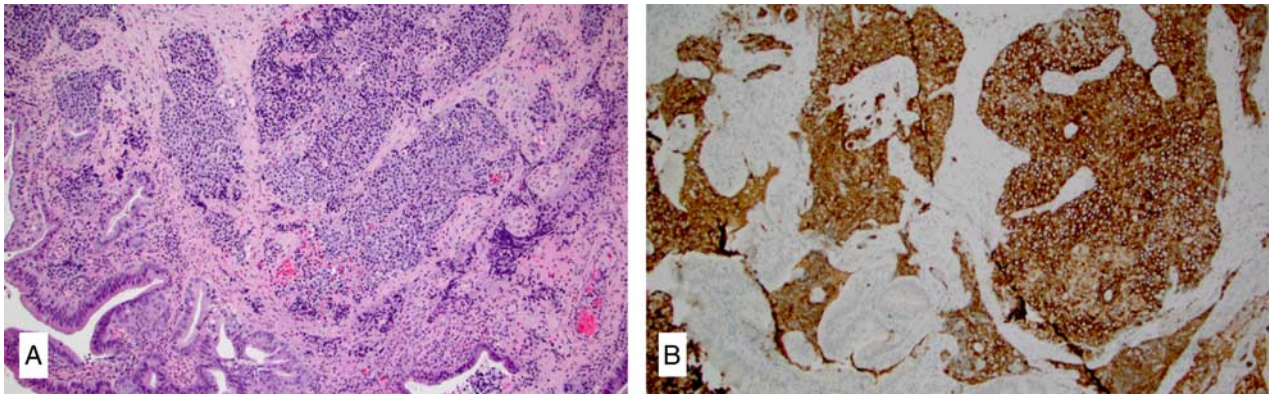

FIGURE 1. Hematoxylin and eosin (H&E) slide (A) of a case of NEC with diffuse and strong DLL3 positivity (B). The majority (81%) of NECs overexpressed DLL3 protein with ~50% of them having diffuse expression (> 50% positive cells).

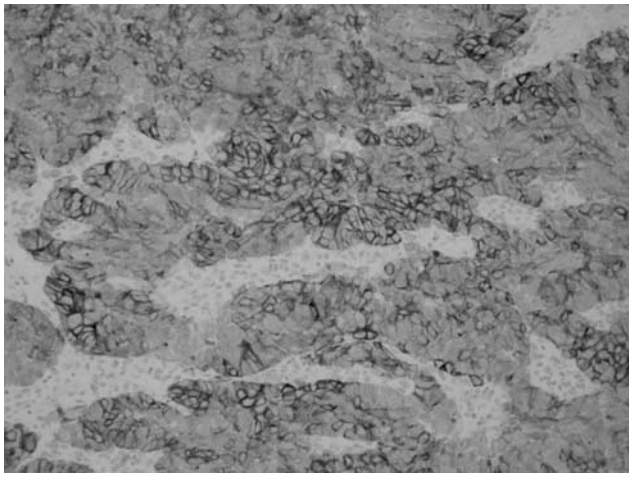

FIGURE 2. A diffuse (> 90% cancer cells) and NTRK expression in a case of cervical strong NEC; however, no *NTRK1-3* gene fusion was seen in this as well as in 9 additional cases that exhibited NTRK expression by immunohistochemistry.

TP53 (11/62), *KRAS* (7/62), *PTEN* (6/62), *CTNNB1* (3/62), *BRAF* (2/62), *RB1* (2/62), *FGFR2* (2/62), and *HNF1A* (2/62), see Table 3 for complete results. *PIK3CA* and *PTEN* mutations were more prevalent in DLL3-negative NECs ($P = 0.018$ and 0.006, respectively). *PTEN* mutations were also more commonly seen in HPV-negative NEC cases ($P = 0.04$). Four cases had concurrent *PIK3CA* and *PTEN* mutations. The mutational profiles did not differ significantly between primary and metastatic NECs.

Copy number alterations were rarely seen in NEC and included amplification of *MYCN* (2/31, 6%), *MCL1*, *MYB*, *MYC*, and *PCM1* (1/31, 3% each).

Gene fusions were not observed in any of the tested cases (n = 31).

Immune-oncology Biomarkers in NEC

PD-L1 positivity (Combined Positive Score ≥ 1) and high-TMB (≥ 17 mutations/Mbp) were significantly lower in NEC compared with SCC of the cervix (10% vs. 79% for PD-L1 and 3% vs. 11% for TMB-high, respectively). Similar to cervical SCCs, all tested NEC cases (n = 31) were MSI stable (Table 4).

TABLE 3. Genomic Profile of Neuroendocrine Carcinoma of the Cervix

Genomic Alterations	Affected Genes in Neuroendocrine Carcinomas of the Cervix
Mutations	*PIK3CA* (11/62, 17%)
	TPPTEN (6/62, 10%)*
	53 (11/62, 17%)
	KRAS (7/62, 11%)
	CTNNB1 (3/62, 5%)
	BRAF (2/62, 3%)
	RB1 (2/62, 3%)
	FGFR2 (2/62, 3%)
	HNF1A (2/62, 3%)
	APC, *AKT1*, *ATM*, *BRCA2*, *CDH1*, *ERBB2*, *ERBB3*, *FBXW7*, *HRAS*, *KDM6A*, *KMT2C*, *KMT2D*, *RET*, *STK11* (single cases each, ~2%)
Copy number alterations	*MYCN* (2/31, 6%)
	MCL1, *MYB*, *MYC*, *PCM1* (1/31, 3%)

*5/5 tested PTEN-mutated cases were human papillomavirus negative.

TABLE 4. Comparison Between NEC and Squamous Cell Carcinoma of the Cervix for 3 Predictive Biomarkers of Response to the Immune Checkpoint Inhibitors (Anti-PD-1/PD-L1)

	n/N (%)	
I-O Biomarkers	Neuroendocrine Carcinoma of the Cervix	Squamous Cell Carcinoma of the Cervix*
PD-L1 positivity	4+/39 (10)	77/98 (79)
TML-H	1/31 (3)	63/599 (11)†
MSI-H	0/31 (0)	6/599 (1)†

*PD-L1 frequency based on KEYNOTE-158 clinical trial [led to FDA approval of pembrolizumab for cervical cancer (2018)]; PD-L1 was tested using 22C3 pharmDx Kit antibody (FDA approved test).

†The data for TMB and MSI status in squamous cell carcinoma were based on the Caris Molecular Profiling results.

FDA indicates Food and Drug Administration; I-O, immune-oncology; MSI-H, microsatellite instability-high; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TML-H, tumor mutational load-high was defined as ≥ 17 mutations/Mbp.

DISCUSSION

NEC is a rare but highly aggressive malignancy with limited therapeutic options, and treatments are extrapolated from studies in other sites. Molecular profiling studies on this cancer are also sparse but the importance of these studies cannot be underestimated given the huge potential benefit if targetable mutations allowed for treatment with emerging drugs.[8,11] Our study is the first to explore biomarkers of response to novel ADCs DLL3, TROP-2, and FOLR1 in an NEC cohort (the ADC drugs and their predictive biomarkers are summarized in Table 2). ADCs are considered one of the most promising anticancer treatments. The therapeutic approach with ADC is based on a monoclonal antibody to a cancer-specific antigen, conjugated to a cytotoxic drug via a chemical linker.[21] This allows cancer cells to be specifically targeted with cytotoxic drugs. Consequently, treatment with ADC substantially diminishes systemic exposure and toxicity.[21] DLL3 protein appears to be the only markedly overexpressed biomarker in our study. Notably, a high DLL3 expression was reported in Merkel cell carcinoma, a variant of highly aggressive cutaneous NEC and small cell lung carcinomas (SCLCs).[22,23] However, a potential therapeutic benefit of DLL3-targeted drugs in NEC remains uncertain given the recent termination of the Rova-T trial after it failed to demonstrate a therapeutic benefit for rovalpituzumab tesirine in patients with SCLC.[24] In our study, DLL3-negative NECs tended to harbor *PIK3CA* and *PTEN* mutations. Both mutations have been commonly described across cancers including NEC and other cervical malignancies,[2,3,8,11] leading to the upregulation of the PI3K-AKT-mTOR signaling pathway.

Interestingly, *PIK3CA* mutations were found in other HPV-associated malignancies such as oropharyngeal or anal cancer.[25,26] These mutations have also been associated with a favorable response to PIK3CA inhibitors, most notably in estrogen receptor–positive breast cancer.[27,28] Therefore, clinical trials exploring the therapeutic effects of PIK3CA/mTOR inhibitors in a subset of NECs are warranted. Similar to our findings, *KRAS* mutations were previously described in NEC[8] although another recent study[29] confirmed only *NRAS* but not *KRAS* mutations in a cohort of 30 NECs. The therapeutic utility of MEK inhibitors in KRAS-mutated NECs remains to be clinically explored.

Oncogenic fusions affecting the *NTRK1-3* genes have been identified in a wide range of human cancers.[13,30] Of cervical malignancies, *NTRK* gene fusions have been described in cervical sarcomas[31,32] and endocervical malignant peripheral nerve sheath tumor.[33] We have also previously reported an *NTRK1* gene fusion in 1/68 cervical SCCs.[13] *NTRK* gene fusions have been described in a small proportion of neuroendocrine malignancies from various anatomic sites including lung (large cell), pancreas, small bowel (with therapeutic response to entrectinib), and uterus.[34,35] Our recent study on breast NEC revealed no *NTRK* gene fusions.[12] The recognition of NTRK-rearranged cancers is clinically relevant given the recent availability of highly effective NTRK inhibitors. The most reliable predictive biomarker for NTRK inhibitors is a gene fusion identified by NGS or other equivalent assay (eg, fluorescence in situ hybridization, reverse transcriptase-polymerase chain reaction).[36] Since we did not identify *NTRK* gene fusions in any of the tested NEC cases including the IHC positive ones, it is unlikely that these patients would gain from the targeted treatment with NTRK inhibitors.

Immune checkpoint inhibitors (against programmed cell death protein 1/PD-L1) have markedly changed the cancer treatment paradigm contributing to better treatment options and improved outcomes for various cancers. The predictive biomarkers for immune checkpoint inhibitors include PD-L1 expression, high tumor MSI and TMB status.[30,37–39] The immune checkpoint inhibitor pembrolizumab/Keytruda has been approved for SCC of the cervix and SCLC. In the case of cervical carcinoma, pembrolizumab is indicated for treatment if the tumor tissue expresses PD-L1 (Combined Positive Score ≥ 1) as determined by a Food and Drug Administration (FDA)-approved test (22C3 pharmDx Kit antibody). Preliminary data based on 2 case studies revealed a substantial therapeutic benefit for nivolumab in 2 NEC patients.[9,10] Notably one of the successfully treated cases was PD-L1 negative, while the other had MSI-high NEC with a high TMB. Nevertheless, our results (PD-L1 expression and high TMB) indicate that ~13% of NEC patients may be candidates for trials with immune checkpoint inhibitors.

In our study, we found that only 43% of the cases were positive for HPV by ISH. The reported rate of HPV in NEC varies and some studies reported HPV positivity in 85% of the cases.[6,40] It is important to emphasize that our ISH cocktail covered high-risk HPVs 16, 18, and 33, while other high-risk HPV types were not covered. Without a history of HPV status at the time of a Pap test, it is difficult to estimate the prevalence of HPV and its importance for the development of NEC. Another limitation of our study is related to the fact that all cases were not explored for all molecular targets; the main reasons for this shortcoming include the lack of remnant tissue samples (for IHC assays) or a poor/insufficient DNA and/or RNA load (tumor mutation load and MSI analyses).

We conclude that patients with NEC have limited targeted therapeutic options. Despite frequent DLL3 expression in NEC, a potential therapeutic benefit for DLL3-targeted drugs remains uncertain given the recent failure of the Rova-T trial in SCLC. A small cohort of NEC enriched in PIK3CA/PTEN pathway mutations, may benefit from PIK3CA inhibitors. Biomarkers of benefit for immune checkpoint inhibition (eg, PD-L1+ and high TMB) were present in ~13% of patients, who consequently may be considered for this therapy.

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