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ORIGINAL ARTICLE

RRM1, ERCC1 and TS1 Immunofluorescence Expression in Leiomyosarcoma: A Tissue Microarray Study with Clinical Outcome Correlation Analysis

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Abstract ERCC1, RRM1 and TS1 are reportedly linked to chemotherapy resistance in lung and other cancers. However, there are currently no studies reporting the relationship between these genes and clinical parameters in leiomyosarcomas.

Method: This study investigated the expression pattern of ERCC1, RRM1 and TS1 in forty-four leiomyosarcoma samples by the use of tissue microarray (TMA), immunofluorescence and AQUA methods. The results were then analyzed for expression level and correlations were made with clinical outcome to determine their potential prognostic value in leiomyosarcoma.

Results: In the forty-four samples studied, the expression level of these three proteins can be well quantified in the AQUA system and reflected by the AQUA score. RRM1 and ERCC1 expression levels did not show any relationship with overall survival. However, a correlation was found between TS1 expression in the cytoplasm and overall survival. The high expression group had a shorter overall survival time (log-rank $p = 0.0498$). This trend was confirmed by the Cox proportional hazards model.

Discussion: The poor overall survival of leiomyosarcoma is linked to TS1 cytoplasm expression which may be useful in predicting prognoses of this tumor, methods targeting expression of TS1 may lead to improved overall survival in leiomyosarcoma, though more detailed information regarding treatment information and a larger sample size is needed to confirm this phenomenon.

Keywords Soft tissue tumor · Leiomyosarcoma · Prognosis · Survival · Tissue microarray · ERCC1 · RRM1 · TS1

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Introduction

Leiomyosarcoma and Molecular Prognostic Factors

Leiomyosarcoma is a malignant smooth muscle neoplasm that can arise in any anatomic location containing smooth muscle, such as uterine tissue, large blood vessels, retroperitoneum, and in the gastrointestinal tract. This neoplasm has also been reported to occur in the skin, bladder, ovaries, salivary glands, larynx, gallbladder, adrenal glands, broad ligament, diaphragm, breast, vulva, penis, scrotum, and testis among others [1–5]. Leiomyosarcoma is rare, comprising less than 1 % of all cancers and less than 10 % of soft tissue tumors. Their occurrence is seen mostly in adults with a peak incidence in the fifth to sixth decade [6]. These aggressive tumors are capable of local recurrence and distant metastasis, most commonly to the lung and less frequently to the liver, brain, bone, pancreas, spinal column, skin, and small bowel [7–10]. Regional lymph node involvement is rare. On histopathological examination, malignancy is mainly determined by tumor necrosis, atypical mitotic activity, and the infiltrative nature. The number of mitoses per 10 high-power fields vary depending on the location of the tumor [11]. Smooth muscle actin,

desmin, caldesmon, smoothelin, and smooth muscle myosin are positive markers in most leiomyosarcoma.

The prognostic factors of leiomyosarcoma include tumor location and tumor size. A retroperitoneal location is fatal in the great majority of the cases due to late detection, large size (>10 cm) at the time of discovery, and difficulty of a complete resection. Leiomyosarcomas arising in blood vessels are also an indication of poor prognosis. The molecular prognostic markers in leiomyosarcoma are active areas of investigation. Studies have found that P53, bcl-2, and Ki-67 are associated with biological aggressiveness [12, 13]. In the era of modern medicine, investigation of biomarkers that have shown prognostic value in other malignancy is a practical strategy to discover additional markers for leiomyosarcoma.

The treatment for leiomyosarcoma is a combination of surgery, radiation, and chemotherapy depending on the tumor stage. The primary role of chemotherapy is in the treatment of metastases, which is a critical determining factor for patients' overall survival time. Conventional chemotherapy includes gemcitabine, doxorubicin, and alkylating agents. The role of targeted therapy is under investigation. The discovery of biomarkers predictive of therapeutic benefit is crucial to improve patients' outcome.

RRM1, ERCC1 and TS1

The RRM1 gene encodes the regulatory subunit of ribonucleotide reductase, the only enzyme capable of converting nucleotides to deoxynucleotides. It is a molecular target of gemcitabine and predictive of treatment efficacy. Upregulation of RRM1 has been observed during DNA repair after chemotherapy and overexpression has been observed in a gemcitabine-resistant human oropharyngeal carcinoma KB clone and in a gemcitabine-resistant human leukemic cell line K562 [14]. In patients with resected NSCLC, increased levels of RRM1 are associated with long survival [15]. In contrast, patients on gemcitabine and platinum therapy for advanced disease have a poor survival if RRM1 expression is high, presumably because of decreased efficacy of chemotherapy [16]. There is high correlation between ERCC1 and RRM1 mRNA levels and a specific effect of RRM1 expression on survival in gemcitabine/platinum-treated stage IV non-small-cell lung cancer (NSCLC) [16–18].

ERCC1 belongs to the family of nucleotide excision repair (NER) genes. It encodes a protein that functions in concert with other members of the repair complex to ensure genomic integrity. ERCC1 participates in DNA damage recognition and DNA strand incision components. The NER pathway is involved in eliminating both platinum DNA adducts and nucleotides damaged by UV-irradiation, and the resulting gaps are filled with precursors for DNA synthesis provided by ribonucleotide reductase. Modified nucleotides, together with adjacent nucleotides, are removed from the damaged strand during the first step (excision), which is followed by synthesis of an intact strand through DNA polymerase (repair synthesis). Overexpression of ERCC1 has been associated with resistance to platinum agents in tumor cell lines, and increased ERCC1 mRNA expression has been shown to predict for platinum resistance and therefore decreased survival in gastric, ovarian, esophageal, and colorectal cancers. In NSCLC, increased expression of ERCC1 is a significant and independent predictor of improved survival in NSCLC patients treated with surgical resection only [19]. These results are consistent with the role of ERCC1 in the repair of modified nucleotides, specifically increased removal of platinum-induced DNA adducts. Hence, in patients with advanced cancers who undergo treatment with platinum-based chemotherapy, increased expression of ERCC1 results in efficient removal of platinum-induced DNA adducts and thus reduced treatment efficacy and survival [17–19].

TS1 is a folate-dependent enzyme that catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate to 2′-deoxythymidine-5′-monophosphate. This pathway provides the sole intracellular de novo source of 2′-deoxythymidine-5′-triphosphate (dTTP, an essential precursor for DNA), and TS inhibition results in depletion of dTTP and an increase in dUTP. This, in time, results in the so-called thymine-less death due to misincorporation of dUTP into DNA; its excision, catalysed by uracil-DNA glycosylase, results in DNA damage. Both this imbalance in dTTP/dUTP and DNA damage can result in induction of downstream events, leading to apoptosis [20, 21]. Therefore, TS1 represents a critical target in cancer chemotherapy.

This study is intended to investigate the expression pattern of ERCC1, RRM1, and TS1 in leiomyosarcoma tissue microarrays (TMA) by an immunofluorescence-based quantitative analysis method. Results are analyzed to determine correlation with clinical outcome and possible potential prognostic value in leiomyosarcoma.

Materials and Methods

This study was carried out in accordance with a research protocol approved by the Institutional Review Board at the Moffitt Cancer Center and the University of South Florida.

Tissue Samples and Patient Data

A retrospective review was conducted to identify previously diagnosed leiomysarcoma patients (1995–2012) archived at the Department of Anatomic Pathology of the Moffitt Cancer Center. The diagnosis was verified by a

sarcoma pathologist (MMB) following the criteria of leiomyosarcoma diagnosis accepted by the World Health Organization via histological examination of H&E slides and confirmation by immunohistochemical or molecular studies. The representative formalin-fixed paraffin-embedded tumor blocks were selected for tissue microarray (TMA) construction. The corresponding H&E slides of the TMA were reviewed to determine the tissue integrity prior to biomarker testing. Pertinent clinical data of these patients were compiled from two sources: 1) the pathology data base to include the patients' age, sex, tumor location, tumor size, and ancillary study results; and 2) the tumor registry to include the patients' treatment and survival information.

Tissue Microarray (TMA) Preparation

A tissue microarray (TMA) is a method for assembling paraffin-embedded tissues from multiple patients into a single block. TMAs consist of cylindrical punches (0.6 mm in diameter) removed from donor paraffin-embedded tissue blocks and re-embedded in a recipient block that contains a regular array of cylindrical holes. The recipient paraffin blocks containing the samples from different donor blocks and can be sectioned to produce more than 100 sections (5 μm each). The resulting TMA sections allow for paralleled immunohistochemical study and quantitative computerized analysis. The advantage of TMAs includes high through-put analysis, decrease assay volume, experimental uniformity, and highly parallel analyses. The leiomyosarcoma TMA was constructed of representative tumor blocks. Inclusion criteria included adequate tumor volume, adequate tissue blocks, and clinical data. Three samples were obtained from different representative areas of a given tumor block and placed adjacent to each other in the TMA.

Immunofluorescence Method

The immunohistochemical (IHC) method used in this study was based on immunofluorescence combined with automated quantitative analysis (AQUA). Immunofluorescence was used to assess in situ expression of the target molecules. Briefly, after being deparaffinized in xylene (3 X 10 min) and rehydrated through graded alcohols to water, the tissue array slides were treated with antigen retrieval solution for 5 min after boiling in a microwave oven, rinsed with wash buffer, treated with 0. 3 % H_2O_2 in water for 15 min, and then blocked for 30 min with 0.3 % BSA. The slides were then incubated at −4 °C overnight with the primary antibody specific for TS1, RRM1, and ERCC1 as in our previous reports [17–20]. Desmin antibodies were used to identify leiomyosarcoma cells. After washing, the slide was incubated with two different secondary antibodies for 1 hour (Envision® labeled polymer-HRP anti-rabbit or Envision® labeled polymer-HRP anti-mouse specific to the first primary antibody, and Alexa 555 goat-anti-mouse or goat-anti-rabbit, based on the source of second primary antibody. 1:200 dilutions in 0.3 % BSA in buffer were used for both). Cy5-Tyramide (1:50 in Amplification solution) was added for ten minutes followed by mounting with Prolong Gold antifade reagent with DAPI mount solution. The slides were scanned with SpotGrabber, and the image data were analyzed with AQUA, which converts the intensity of the fluorescent signal of the antibodies bound to the target antigens into quantitative digital data (PM-2000, HistoRx, New Haven, Conn).

Statistical Analysis

The actual values for the AQUA scores of TS1, RRM1, and ERCC1 for replicate specimens were averaged, treated as independent continuous variables, and analyzed using the proportional hazards regression test. The primary objective was to assess the association between in situ protein expression of the above biomarkers and overall survival (OS). For this analysis, patients were dichotomized into high and low target expression groups using the mean values as cutoffs.

Results

Clinical and Pathologic Characteristics

Forty-four individual patient specimens were collected for TMA construction and AQUA analysis. The mean age was 57.1. 15 of the patients were men and 29 were women. OS was defined as the time elapsed from the date of diagnosis to the date of death. The vital status of patients was verified using the publically available National Social Security Death Index (SSDI). If not found, patients were censored as 'alive' as of January 15th, 2013, which was approximately 1 months before this analysis started or as of a later date if supported by their medical record. The range of follow up was 0.15 to 18.21 months (mean 4.98 months). Other characteristics of the 44 eligible patients: median age of 58 (range: 26 to 85) years old, male to female ratio of 15:29, tumor size median of 8.5 (range: 1.3–25) cm.

Characteristics of RRM1, ERCC1, and TS1 Expression

In situ localization detected RRM1 and ERCC1 in the nucleus, while TS1 was found in both the cytoplasm and nucleus in leiomyosarcoma cells. The tumor stroma showed no evidence for signal detection by the three antibodies, which is a result of using making technology

Table 1 AQUA Scores of RRM1, ERCC1 and TS1 Staining

AQUA Score	Minimum	Maximum	Mean	SD
RRM1 (nucleus)	0.00	1334.94	540.88	277.42
ERCC1 (nucleus)	260.83	1320.24	582.95	226.91
TS1 (cytoplasm)	114.61	1603.84	1017.68	492.20
TS1 (nucleus)	211.21	1428.22	756.08	405.36

through the second primary antibody and consistent with other previously reported investigations. The mean values of AQUA scores as shown in table 1.

Association of ERCC1, RRM1 and TS1 Expression with Overall Survival

The primary endpoint of this analysis was OS based on high vs. low expression of ERCC1, RRM1, and TS1; the mean AQUA scores of ERCC1, RRM1 and TS1 were used as the cutoff to designate high or low expression level. The median follow-up time was 4.1 years, and the maximum was 18.2 years. We found no difference in OS for patients with high compared to low ERCC1 or RRM1 expression (Fig. 1a, b). Similarly, patients with high versus low nuclear TS1 expression had indistinguishable OS (Fig. 1c); however, there

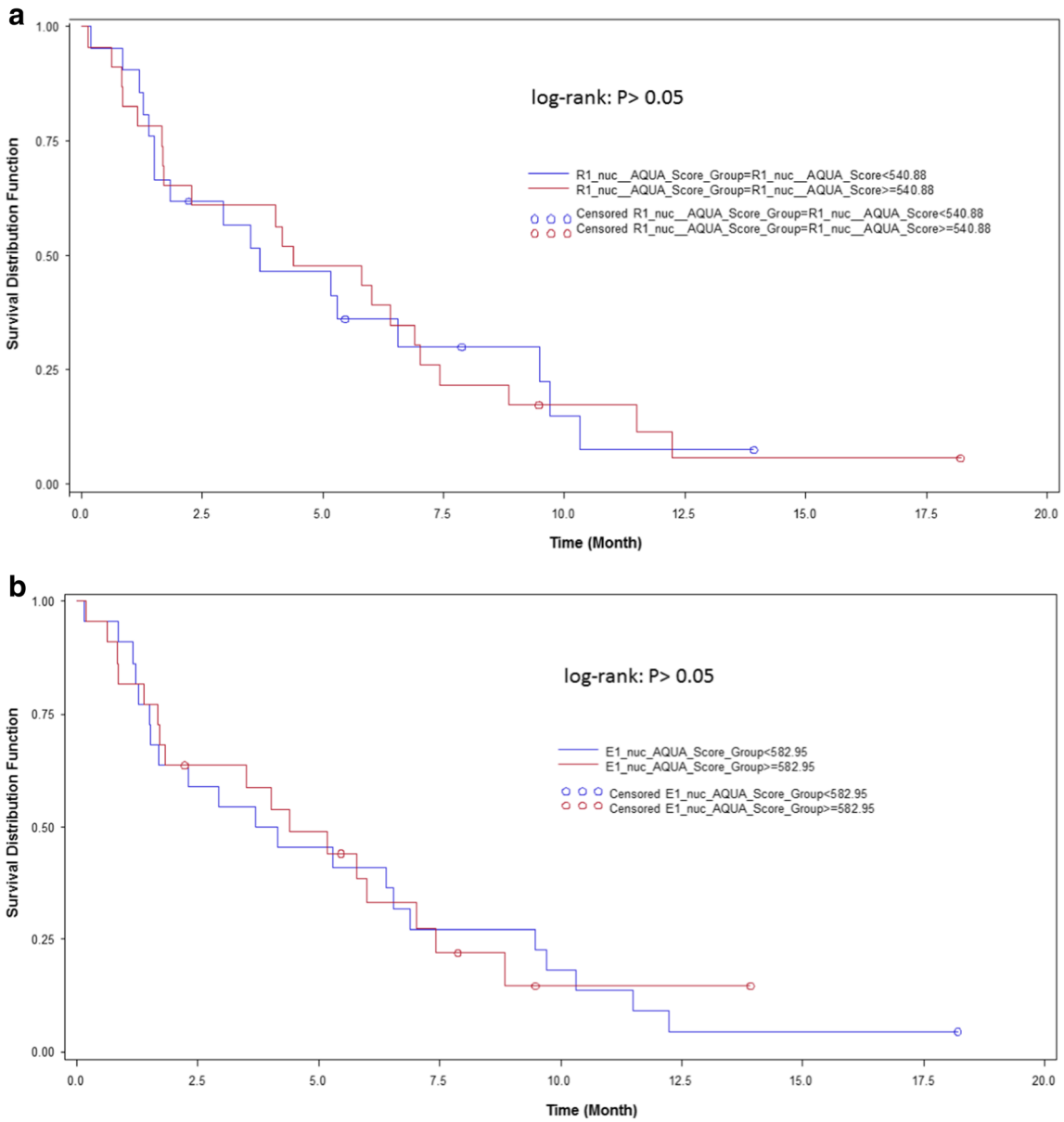

Fig. 1 Overall Survival Analysis of RRM1 (panel **a**), ERCC1 (panel **b**), TS1 (panel **c** and **d**). The data shows that Expression of RRM1, ERCC1 and TS1 nuclear staining level is not correlated with patients overall survival time. But the cytoplasm expression level does (see panel d): based on AQUA score cutoff, those patients with lower expression level of cytoplasm TS1 have better OS time comparing to the higher expression level group

was a statistically significant (log-rank $p = 0.0498$) survival advantage for patients with low compared to high cytoplasmic TS1 expression (Fig. 1d). In an analysis of clinicopathological characteristics with OS, we found that men had a longer survival then women (log-rank $p = 0.05$), and all other parameters had no significant survival association.

These results were confirmed in a Cox proportional hazards model analysis that included the parameters ERCC1, RRM1, TS1, age, and gender. We found that only gender and cytoplasmic TS1 expression were associated with OS; high cytoplasmic TS1 and female gender had increase hazards of death. There was interaction between covariants (Table 2). In this analysis one SD increase in TS1 cytoplasm levels will increase the log odds of hazard by 0.37837 ($p = 0.05$).

Table 2 Multivariate analysis: Cox proportional hazard model

Variable	Parameter Estimate	SD	Hazard Ratio	*p Value*
Age	0.00295	0.01375	1.00	0.83
Gender (F vs M)	0.72116	0.40193	1.76	0.07
RRM1	−0.08939	0.35389	0.91	0.80
ERCC1	−0.03952	0.35235	0.96	0.91
TS1 Cyto	0.60425	0.37837	1.83	0.05
TS1 Nuc	−0.13912	0.35678	0.87	0.70

Discussion

Leiomyosarcoma is an aggressive sarcoma that can arise in a number of locations [3]. It remains one of the most difficult to

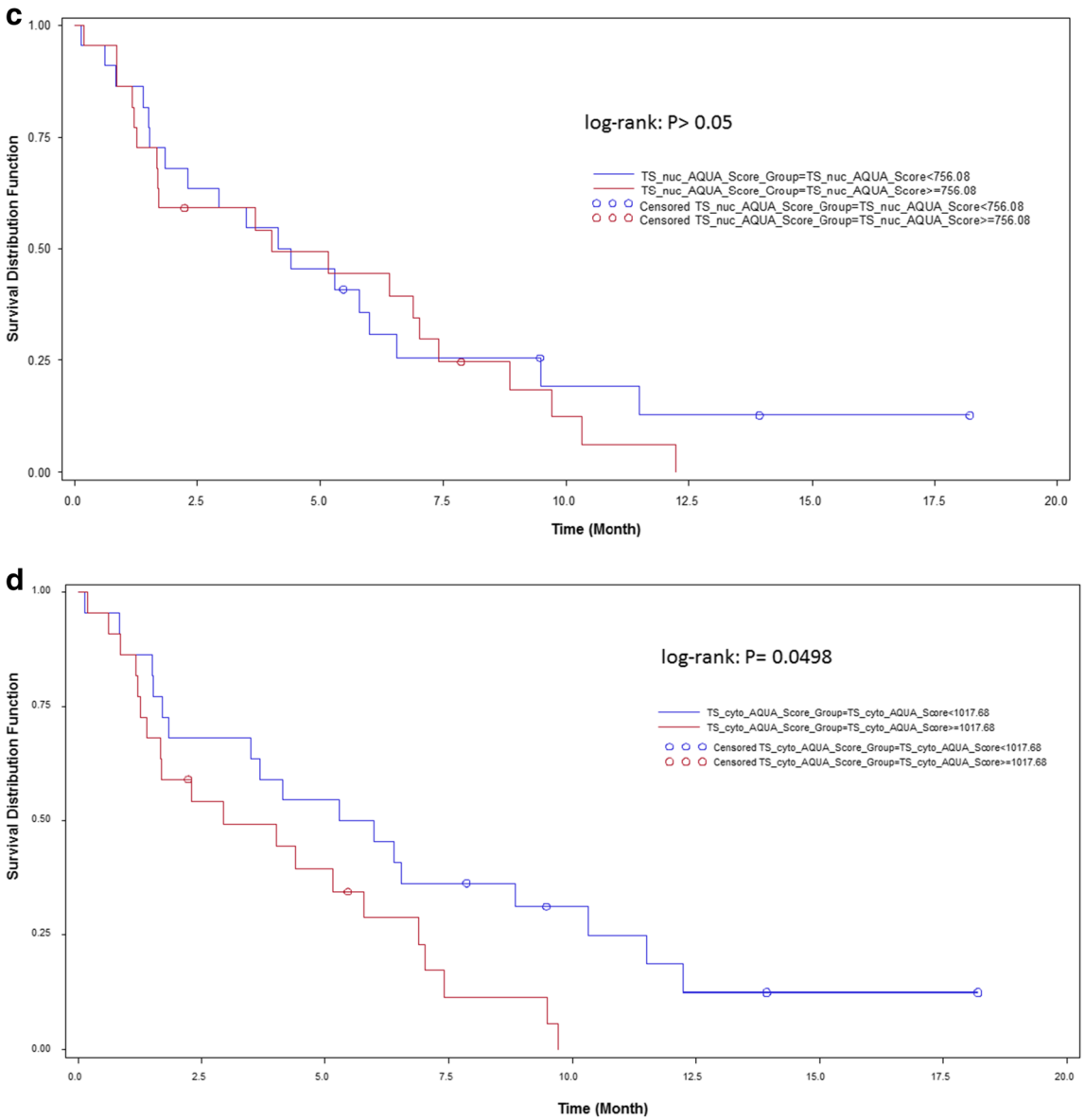

Fig. 1 (continued)

treat soft-tissue sarcomas, and the discovery and validation of prognostic and predictive biomarkers is likely to facilitate the development of more effective therapies.

RRM1, ERCC1, and TS1 expression levels have been studied in lung cancer and other tumors with the goal to guide physicians and patients in therapeutic decision making. Our data, based on tumor specimens from forty-four patients, show that nuclear expression levels of ERCC1, RRM1 and TS1 cannot be used as prognostic biomarkers for OS. However, high expression of cytoplasmic TS1 may be indicative of poor overall survival. However, the number of patients investigated is relatively small (N = 44), which lessens the strength of evidence for our conclusions. We recommend that cytoplasmic TS1 levels be investigated in larger and independent datasets of patients with leiomyosarcoma to obtain additional corroborating data before engaging in a large-scale validation study. Given the world-wide availability of nucleoside analogues and folate antagonists for cancer treatment, validation of TS1 may subsequently lead to use of this biomarker for prediction of therapeutic efficacy.

A known mechanism of resistance to TS1 inhibitor compounds is drug-mediated acute induction of TS1 synthesis. This mechanism is directly controlled at the translational level, and it has been associated with resistance to pemetrexed. The future success of TS1 inhibitor compounds in the clinic may depend on novel strategies to selectively inhibit TS1 and on novel combination therapies to overcome cellular drug resistance [21]. Our preliminary results indicating a survival association with TS1 expression in patients with leiomyosarcoma suggests that TS1 expression should be considered when conducing future trials with such agents.

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