

CHAPTER III

**THE IMPORTANCE OF A NEW TUMOR MARKER TRA-1-60 IN THE
FOLLOW-UP OF PATIENTS WITH CLINICAL STAGE I
NONSEMINOMATOUS TESTICULAR GERM CELL TUMORS**

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ABSTRACT

TRA-1-60 is a new tumor marker for embryonal carcinoma-positive non-seminomatous testicular germ cell tumors (NSTGCT). Upper normal reference value (RV) and serum half-life time ($T_{1/2}$) were determined. The value was determined in the follow-up of 154 patients with stage I NSTGCT. TRA-1-60 was measured in normal controls (n=100) to determine RV and in patients without recurrence for $T_{1/2}$. In all patients TRA-1-60 was determined at the time of orchidectomy. In 42 patients with recurrence, values were also evaluated one month before and at the time of CT-confirmed recurrence. Predictive values and survival probability were examined and compared to AFP and hCG. RV was 230 U/ml and $T_{1/2}$ 9.5 days. Elevated TRA-1-60 at the time of orchidectomy was not associated with recurrence. One month before recurrence, 21 of 42 patients had elevated TRA-1-60 levels (50%); 10 were negative for both AFP and hCG. At the time of recurrence, 24 patients had elevated TRA-1-60 levels (57.1%); nine were negative for AFP/hCG. Patients with TRA-1-60 levels of >500 U/ml had a poorer recurrence-free survival probability ($P=.015$). TRA-1-60 is useful in the follow-up of stage I NSTGCT. The combination of AFP, hCG and TRA-1-60 may improve the early detection of recurrence.

INTRODUCTION

For many years, it has been known that nonseminomatous testicular germ cell tumors (NSTGCT) may secrete human chorionic gonadotrophin (hCG) and alpha-fetoprotein (AFP), which are now the principal testicular serum tumor markers in widespread clinical use. Diagnosis, staging and follow-up of patients with these tumors have been aided considerably by assaying these markers.¹⁻⁵ In the past few years, the patterns of secretion before, during and after treatment have been extensively studied and described.⁶⁻⁹ Some understanding of the underlying biological implications of marker production has been reached. Despite this, the use of AFP and hCG is not ideal, because at least 20% of the patients with an NSTGCT do not produce these markers.¹⁰ Therefore, in recent years, much effort has been devoted to searching for new tumor markers.

In 1984, Andrews et al. found that the Terato Related Antigen monoclonal

antibody (TRA-1-60), raised against embryonal carcinoma cells, recognizes a high-molecular-weight glycoprotein that is expressed on the surface of embryonal carcinoma progenitor cells.^{11,12} In 1991, Marrink et al. developed a serum immunoenzymometric assay for the detection of a TRA-1-60 reactive antigen.¹³ This antigen is released into the serum of patients with embryonal carcinoma-positive NSTGCT.

The purpose of this study was to determine whether the TRA-1-60 antigen has any additional value as a tumor marker to hCG and AFP in the follow-up of patients with clinical stage I NSTGCT. The upper normal reference value and the serum half-life of TRA-1-60 were determined. It was also investigated whether an elevated level of TRA-1-60 at the time of unilateral orchidectomy was an unfavourable prognostic factor that might be related to recurrence.

PATIENTS AND METHODS

At the University Hospital Groningen, the Netherlands, serum samples were obtained from all consecutive patients with clinical stage I NSTGCT treated according to the wait-and-see policy between 1982 and 1992.¹⁴ All the patients underwent orchidectomy and did not have any evidence of regional and/or distant metastases at clinical staging. Clinical staging was performed as described by Peckham et al,¹⁵ with the aid of physical examination, chest X-rays, tumor marker assays of AFP and hCG and CT scans of the chest and abdomen. Tumors were classified in accordance with the nomenclature of the World Health Organisation;¹⁶ the pT stage was determined according to 1978 International Union Against Cancer criteria.¹⁷ Outpatient follow-up evaluations were performed according to a strict schedule and serum AFP and hCG determinations were performed at each visit. After ten years, the follow-up period was considered to be complete and patients were discharged from any further evaluations. During this period, 42 out of the 154 patients had developed a recurrence, all within two years of the orchidectomy.¹⁴ These patients were therefore treated with cisplatin-based combination chemotherapy.^{18,19}

Serum samples were obtained before and/or as soon as possible after orchidectomy from all 154 patients for the determination of tumor marker concentrations. TRA-1-60 levels were included in the statistical analyses if they

had been determined from serum samples obtained before or ≤ 10 days after orchidectomy. From the 42 patients who developed a recurrence, serum samples were also obtained at the time that recurrence was confirmed by CT and one month before recurrence. In the patients who had elevated levels of TRA-1-60 at the time of recurrence, TRA-1-60 levels were also determined during and after polychemotherapy treatment. TRA-1-60 values were compared to those for AFP and hCG determined in the same serum samples.

In the TRA-1-60 assay, the antigen was incubated with excess TRA-1-60 antibody, after which the antigen/antibody complex was precipitated by 2.5% polyethylene-glycol (PEG). The non-bound antibodies in the supernatant were subsequently transferred to antigen-coated wells in a microtiter plate. The amount of unbound antibody was then quantified by its ability to bind to the coated antigen as detected by a peroxidase-coupled second antibody.^{13,20} Absorbance was measured at 492 nm using a Titertek Multiscan (Flow, Irvine, UK). Thus, in this indirect assay, the extinction measured was inversely related to the amount of antigen present in the original sample. Results were computed according to Rodbard et al²¹ and quantified in arbitrary units per millilitre (U/ml). To determine the upper normal reference value, TRA-1-60 was measured in a healthy male population of normal controls (n=100), age-matched to the tumor population. Logarithmic transformation (natural logarithm) of TRA-1-60 levels was performed to approximate a normal distribution. The upper normal reference value was calculated by taking the antilog of the geometrical mean, i.e. antilog of mean \ln TRA-1-60, plus twice its standard deviation.^{22,23} Serum AFP and serum hCG were determined by immunoassay.²⁴ AFP and hCG values were considered to be elevated at $>10 \mu\text{g/l}$ and $\geq 2 \mu\text{g/l}$, respectively.

To determine the serum half-life ($T_{1/2}$) of TRA-1-60, serum samples were obtained from patients with a clinical stage I NSTGCT without recurrence. Only the serum samples of patients who had elevated levels of TRA-1-60 of $>850 \text{ U/ml}$ and elevated levels of AFP and/or hCG were used. From the patients with elevated levels of AFP and/or hCG, samples for tumor marker assays were obtained two or three times a week after orchidectomy until normalisation. During follow-up, AFP and hCG determinations were performed every four weeks. If the AFP and/or hCG levels in these sera normalized postoperatively in exact accordance with the half-life for AFP of 6 days and hCG of 2 days, it was assumed that the TRA-1-60 levels had also normalized in accordance with its half-life time. The TRA-1-60 half-life time was calculated using the method described by Lange et al.²⁵

Statistical analysis

Log transformation (natural logarithm) was performed on tumor marker levels, to approximate a normal distribution. Geometric means were calculated by taking the antilog of the means of these log-transformed tumor marker levels.

Differences in mean tumor marker levels between the two subgroups of patients with recurrence and those without recurrence were tested by means of the Student *t*-test in the case of continuous variables. Differences in dichotomous variables between the two subgroups of patients with recurrence and those without recurrence were tested by means of the Chi-square test. Fisher's exact test was used in the case of small numbers.²³

Recurrence-free survival probabilities of the subjects with normal and those with elevated tumor marker levels were determined with Kaplan-Meier curves.

Differences in survival probabilities were analysed by the logrank test. Follow-up started at the time of orchidectomy and ended at the time of CT-confirmed recurrence in the patients with recurrence. In the patients without recurrence, follow-up ended ten years after orchidectomy or on 1 January 1995. Cox regression analysis was used to evaluate the independent associations between elevated tumor markers and recurrence, adjusted for the influence of other, previously published covariates: age, tumor location (left or right), histological components of the primary tumor, presence of vascular invasion in the primary tumor, and the pT stage.⁹ Age and tumor markers were used as continuous variables, while the remaining variables were dichotomized.

Differences were considered to be statistically significant at $P < .05$. Hazard ratios were considered to be statistically significant if the 95% confidence interval did not contain the value 1. The statistical analyses were conducted using the SPSS-PC+ (version 4.0) software package (SPSS Inc, Chicago, IL, USA).

RESULTS

The mean TRA-1-60 antigen level in the healthy controls was 77.0 U/ml, median 61.5 U/ml (range 20-268 U/ml, $n=100$). The geometric mean was 61.9 U/ml, standard deviation 1.9 U/ml. The upper normal reference value for TRA-1-60 was calculated and after back-transformation, established at 230 U/ml (antilog of $\ln 61.9 + 2 \times \ln 1.9$).

In 65 out of the 154 patients, serum samples were available from before and/or ≤ 10 days after orchidectomy. A total of 35 of these 65 patients had an elevated level of AFP and/or hCG. Ten of these patients developed a recurrence, which left 25 patients with elevated levels of AFP and/or hCG without recurrence. From these 25 patients, five also had TRA-1-60 levels of > 850 U/ml in the first serum sample. The serum samples of these five patients were used to determine the $T_{1/2}$ of TRA-1-60; the median follow-up duration was 10 years, range 7 to 11 years. The mean serum half-life time was 9.5 days, median 9.5 days (range 8.5 - 10.5 days).

In the 65 patients whose serum samples were available at the time of orchidectomy, 17 patients developed a recurrence. In the remaining 48 patients without recurrence, one patient with a very high level of AFP (970 $\mu\text{g/l}$) and one patient with a very high level of hCG (1217 $\mu\text{g/l}$) were considered as outliers and were excluded from the analyses. Statistical significance was not influenced by this. Thus, our analyses were performed on a population of 63 patients: 17 with recurrence and 46 without recurrence.

Tables 1a and 1b list comparisons of the tumor markers AFP, hCG and TRA-1-60 in the study population, stratified by the recurrence status. For all the tumor markers, the geometric mean was higher in the patients with recurrence than in those without. However, these differences were not statistically significant (Table 1a).

Table 1a. Tumor markers at the time of orchidectomy in relation to the recurrence status (continuous)

Tumor marker	No Recurrence n=46	Recurrence n=17	P
Geometric mean			
AFP ($\mu\text{g/l}$)	10.8	18.3	.243
hCG ($\mu\text{g/l}$)	1.0	1.5	.309
TRA-1-60 (U/ml)	223.4	328.4	.089

Dichotomous comparison showed that the proportion of elevated tumor marker levels was higher in the patients with recurrence than in those without. Again, none of these differences were statistically significant (Table 1b).

Table 1b. Tumor markers at the time of orchidectomy in relation to the recurrence status (dichotomous)

Tumor marker	No Recurrence n=46	Recurrence n=17	P
Tumormarker elevated			
AFP (%)	21 (45.7)	10 (58.8)	.353
hCG (%)	7 (15.2)	5 (29.4)	.279
TRA-1-60 (%)	23 (50.0)	10 (58.8)	.534

Predictive values of the tumor markers AFP, hCG and TRA-1-60 in these 63 patients are compared in Table 2.

Table 2. Predictive values of the tumor markers

Characteristic	AFP	hCG	TRA-1-60
Sensitivity	0.59	0.29	0.59
Specificity	0.54	0.85	0.50
Positive Predictive Value	0.32	0.42	0.30
Negative Predictive Value	0.78	0.76	0.77

Serum TRA-1-60 and AFP had a similar sensitivity (0.59) and specificity (0.50; 0.54). Serum hCG had a low sensitivity (0.29) with a high specificity (0.85). Positive and Negative Predictive Values for these three tumor markers were basically the same. Thus, AFP and TRA-1-60 best detected disease and hCG best detected the absence of disease in this group of patients with clinical stage I NSTGCT. However, overall AFP, hCG and TRA-1-60 were of comparable value.

The influences of AFP, hCG and TRA-1-60 on the recurrence-free survival probability were determined by means of Kaplan-Meier survival analyses. Patients with elevated levels of TRA-1-60 of >230 U/ml in the first serum sample did not have a significantly poorer recurrence-free survival probability. The same was true for elevated levels of AFP and hCG. Only the patients with elevated levels of TRA-1-60 of >500 U/ml proved to have a significantly poorer recurrence-free survival probability ($P=.015$ in logrank test). Six out of the 17 patients with recurrence (35.3%) and five out of the 46 patients (10.9%) without recurrence had elevated levels of TRA-1-60 of >500 U/ml.

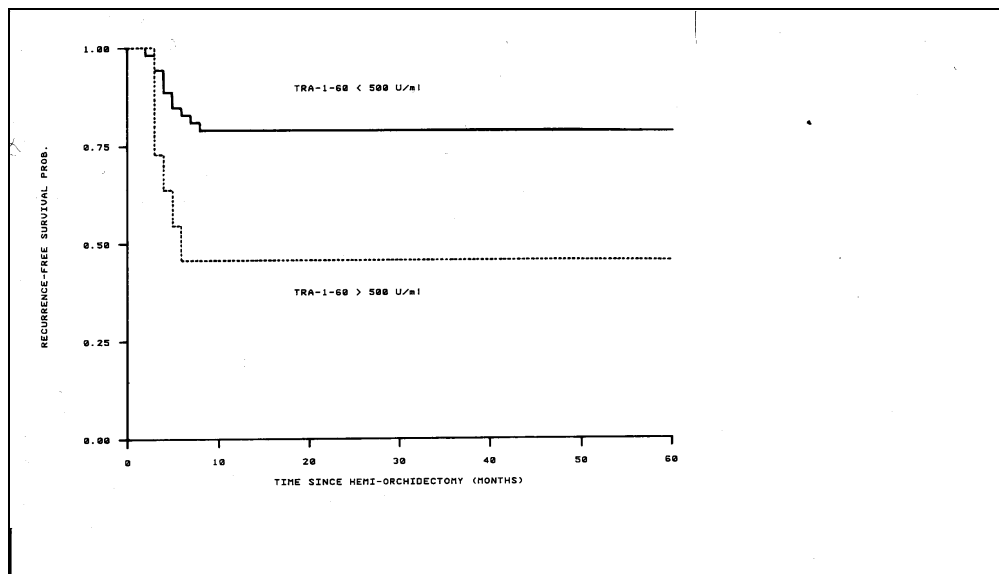


Figure 1. Recurrence-free survival probability in 52 patients with a TRA-1-60 of <500 U/ml compared to 11 patients with a TRA-1-60 of >500 U/ml (X^2 5.9; $P=.015$)

Figure 1 shows the Kaplan-Meier curves for the 52 patients with a TRA-1-60 of <500 U/ml, compared to the 11 patients with a TRA-1-60 of >500 U/ml. However, in the multivariate comparison by means of Cox regression analysis, no independent association was found between a TRA-1-60 of >500 U/ml and recurrence (Hazard Ratio 2.5, 95% Confidence Interval 0.5-13.1).

Table 3 shows the proportion of elevated tumor markers in the sera of the 42 patients who developed a recurrence. Tumor marker levels were determined in the first available serum sample, which was obtained before or less than ten days after orchidectomy (I; n=17) and in the samples obtained one month before CT-confirmed recurrence (II; n=42) and at the time of CT-confirmed recurrence (III; n=42).

Table 3. Elevated tumor markers in patients with recurrence

Tumor marker positive	I (n=17)		II (n=42)		III (n=42)	
	No.	%	No.	%	No.	%
TRA-1-60	10	58.8	21	50.0	24	57.1
AFP	10	58.8	8	19.0	14	33.3
hCG	5	29.4	11	26.2	19	45.2
AFP and/or hCG	10	58.8	18	42.9	26	61.9
AFP a/o hCG a/o TRA-1-60	13	76.5	28	66.7	35	83.3

I, at the time of unilateral orchidectomy; II, one month before recurrence; III, at the time of recurrence.

In the first serum sample, ten out of the 17 patients had an elevated level of TRA-1-60 (58.8%), ten patients had an elevated level of AFP (58.8%) and five patients had an elevated level of hCG (29.4%). Ten patients had an elevated level of AFP and/or hCG (58.8%), while 13 patients had elevated levels of AFP and/or hCG and/or TRA-1-60 (76.5%). Three of the TRA-1-60 positive patients were negative for the conventional tumor markers.

One month before recurrence, 21 patients already had elevated levels of TRA-1-60 (50.0%), whereas only 8 patients had elevated levels of AFP (19.0%) and 11 patients had elevated levels of hCG (26.2%). Ten of the 21 TRA-1-60 positive patients were negative for both AFP and hCG. At the time of CT-confirmed recurrence, 24 out of the 42 patients had an elevated level of TRA-1-60 (57.1%), while 26 patients (61.9%) had elevated levels of AFP and/or hCG. Nine out of the 24 patients with elevated levels of TRA-1-60 were negative for both AFP and hCG. TRA-1-60 levels were elevated more often one month before recurrence and at the time of recurrence than the conventional tumor markers AFP and hCG. During polychemotherapy treatment all the elevated tumor markers normalised.

DISCUSSION

AFP and hCG are currently in routine use for the diagnosis, staging and follow-up of patients with NSTGCT. AFP is produced by yolk sac tumor components and undifferentiated embryonal carcinoma cells,²⁶ as well as by immature or mature teratoma.²⁷ HCG staining is present in choriocarcinoma and syncytiotrophoblastic giant cells,²⁶ but also in undifferentiated mononuclear embryonal carcinoma cells.⁹ Lactate dehydrogenase (LDH) has been suggested as a potential marker, but it only appears to be reliable in bulky disease. LDH rarely provides additional information to that of serum hCG or AFP, either during therapy or monitoring.²⁸ Carcinoembryonic antigen (CEA) offers some advantage in that it is elevated in the undifferentiated NSTGCT consisting of mature teratoma.²⁹ In 80% of the NSTGCTs, at least one of these serum tumor markers is increased.⁹ Nevertheless, conventional tumor markers are liable to fail in a substantial number of patients because NSTGCTs generally contain various histological components and cell types that differ in their ability to synthesize one or more markers. Embryonal carcinoma is the most prevalent histological component in NSTGCT; about 70% contain this component.³⁰ Because the TRA-1-60 antigen is released into the serum of patients with embryonal carcinoma-positive NSTGCT, it is an important potential tumor marker.

TRA-1-60 was measured in 100 healthy male controls, age-matched to the tumor population. The upper normal reference value for TRA-1-60 was calculated and established at 230 U/ml.

In a former publication, we described the histology of the primary tumors in this group of patients with clinical stage I NSTGCT: 132 out of the 154 primary tumors (85.7%) were embryonal carcinoma-positive.¹⁴ In the present study, serum samples were available at the time of orchidectomy in 65 out of these 154 patients; 56 (86.1%) of the primary tumors in these patients contained an embryonal carcinoma component, while 30 out of these 56 patients (53.6%) had elevated levels of TRA-1-60 in the initial serum sample. However, four of the patients with elevated levels of TRA-1-60 did not have an embryonal carcinoma component in the primary tumor. It is possible that an embryonal carcinoma element in the primary tumor was overlooked during histological examination.

The metabolic clearance rate is expressed by the half-life time of a serum tumor marker. Several authors have proposed that the rate at which the serum tumor marker levels decrease during or after treatment could have prognostic significance.^{2,6,7,31,32} If any marker-producing tissue remains in situ, the levels can be expected to decrease more slowly because the markers would continue to be released into the circulation.^{8,25,33} For the calculation of the $T_{1/2}$ of TRA-1-60, the serum samples of five patients without recurrence who had TRA-1-60 levels of >850 U/ml in the initial serum sample were used. Mean $T_{1/2}$ was 9.5 days (range 8.5-10.5 days). The $T_{1/2}$ of TRA-1-60 is longer than the half-life time of six days for AFP and of two days for hCG. TRA-1-60 may be at a slight disadvantage because of this. However, the interval between tumor marker determination and orchidectomy was usually more than one week. Therefore it can be expected that any increase or decline in TRA-1-60 levels would have been noted.

Marrink et al. found that TRA-1-60 was elevated more often (76%) than AFP (57%) or hCG (38%) in patients with disseminated NSTGCT and embryonal carcinoma in the primary tumor.¹³ They described that in 10 out of their 15 patients in whom the AFP and hCG levels were both within the normal range, the TRA-1-60 level was elevated. Therefore, it was the only detectable serum tumor marker released by the embryonal carcinoma cells. Our study population consisted of patients with clinical stage I NSTGCT only. In the patients with recurrence, TRA-1-60 was the only detectable serum tumor marker in three out of the 17 patients at the time of orchidectomy (17.6%). One month before recurrence, it was the only elevated marker in ten out of the 42 patients (23.8%); at the time of recurrence, it was the only elevated serum tumor marker in nine out of the 42 patients (21.4%). Therefore, TRA-1-60 proved to be of additional

value to AFP and hCG in the follow-up of patients with clinical stage I NSTGCT.

Discordance in the behaviour of AFP and hCG has been described by other investigators^{5,34-36} and has also been reviewed.³⁷ This discordance presumably reflects irradiation of the marker-producing portion of the tumor cell population with survival and recurrence of another portion.³⁸ TRA-1-60 also seems to display discordant behaviour: in this study, elevated values of TRA-1-60 were found in the initial serum samples of ten out of the 17 patients who developed recurrence. However, at the time of recurrence only seven of these patients had elevated TRA-1-60 levels. The opposite was observed in four patients who had normal serum TRA-1-60 levels in the first serum sample but elevated levels at the time of recurrence. Although the TRA-1-60 levels generally follow the AFP/hCG curves, the three markers sometimes diverge during follow-up, which provides synergistic information for the management of patients with clinical stage I NSTGCT.¹³

Other studies have shown that the incidence and perhaps the level of tumor markers have prognostic significance.^{37,39,40} In our population, no significant differences in recurrence-free survival were found in the initial tumormarker levels of AFP, hCG and TRA-1-60 at cut-off point of 230 U/ml between the patients with and those without recurrence. Only the patients who had elevated levels of TRA-1-60 of more than 500 U/ml had a significantly poorer recurrence-free survival probability ($P=.015$). Sensitivity, specificity and predictive values of TRA-1-60 in the initial serum samples were similar to those of AFP and hCG. If, on the basis of clinical, biochemical and pathological criteria, patients at high risk of developing metastases could be identified prospectively at the time of orchidectomy, then consideration could be given to administering immediate adjuvant treatment with chemotherapy.^{3,41,42} We have reported previously on the wait-and-see policy for patients with clinical stage I NSTGCT.¹⁴ In multivariate logistic regression analyses, recurrence was found to be related to the presence of vascular invasion, embryonal carcinoma, an elevated preoperative hCG level and the absence of mature teratoma. Only vascular invasion was an independent unfavourable prognostic risk factor for developing recurrence. On the basis of these data, the question arises as to whether a combination of an elevated level of TRA-1-60 of >500 U/ml and vascular invasion of the primary tumor are predictive of recurrence. In our 17 patients with recurrence, five (29.4%) had vascular invasion and an elevated level of TRA-1-60 of >500 U/ml. In the 46

patients without recurrence, there were two patients who had both characteristics (4.3%). Thus, 5 (71.4%) out of the seven patients with both characteristics developed recurrence. Compared to the patients without elevated tumor markers and without vascular invasion, all the patients with a combination of an elevated level of one of the tumor markers at the time of orchidectomy and vascular invasion, had a significantly poorer recurrence-free survival probability (Table 4). This was valid for univariate as well as for multivariate comparison. However, these results were obtained from a group of 63 patients with a relatively small number of recurrences (n=17) and should therefore be interpreted with caution. If additional studies of a larger number of patients confirm these results, it may be justified to offer adjuvant treatment to patients with a clinical stage I NSTGCT who have a combination of vascular invasion of the primary tumor and an elevated level of one of the tumor markers AFP, hCG or TRA-1-60. However, the administration of adjuvant polychemotherapy to all of our patients with these characteristics would have meant the overtreatment of 2 out of the 7 patients (28.6%).

Table 4. Differences in recurrence-free survival probability of patients with an elevated tumor marker and vascular invasion (vasc), compared to patients without these characteristics

	Univariate P	Multivariate P
AFP+ and vasc+	.002	.016
hCG+ and vasc+	.002	.010
TRA-1-60+ and vasc+	.007	.039
TRA-1-60 >500 U/ml and vasc+	.001	.006

These data indicate that the TRA-1-60 antigen, as a serum tumor marker in the follow-up of patients with clinical stage I NSTGCT, provides useful additional information. With the aid of TRA-1-60 we may be able to detect recurrences earlier, especially in patients who are negative for both AFP and hCG. The TRA-1-60 level in the initial serum samples taken at the time of orchidectomy is

not an independent risk factor for recurrence. However, univariate comparison showed that TRA-1-60 elevation of more than 500 U/ml was an unfavourable prognostic factor. If confirmed in other studies, it might be feasible to add TRA-1-60 assays to the follow-up evaluation schedules of patients with NSTGCT.

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