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Modified Ribonucleosides as Biological Markers for Patients with Small Cell Carcinoma of the Lung*

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Abstract—*A variety of individual modified ribonucleosides may be elevated in the urine of cancer patients. They can be readily measured quantitatively in a single reversed-phase high-performance liquid chromatographic run. A total of* 41 *patients with small cell carcinoma of the lung were studied. For* 5*-ribonucleosides determined in the pretreatment urine of* 28 *patients, the respective frequency of elevation was directly related to stage of disease. One or more nucleosides were evaluated in the pretreatment urine of* 27 *out of* 28 *patients* (96%). *Included were* 11 *patients with limited disease and* 10 (91%) *had* 2 *or less than* 2 *nucleosides elevated, whereas* 16 *out of* 17 (94%) *with extensive disease had* 3 *or more elevated. Based on this same discriminant, median survival was significantly extended for patients with* 2 *or less nucleosides elevated* (24 months) *in contrast to* 3 *or more* (10 months). *Using a single number to represent the summation of equally weighted individual nucleoside values as a composite score, a direct relationship was found between increasing extent of disease or tumor burden. This was in contrast to more variable results for carcinoembryonic antigen analyzed in plasma samples obtained at the same time. When determined serially the composite score paralleled in general the clinical response categories for individual patients.*

INTRODUCTION

MODIFIED ribonucleosides, predominantly degradation products of transfer ribonucleic acid (tRNA), have been shown to be excreted in abnormal amounts in the urine of cancer patients [1–7]. Interest in these materials as potential biological markers (biomarkers) was stimulated following evidence that tRNA methyltransferase from cancer tissue had both increased activity and capacity when compared to the enzyme derived from the corresponding normal tissue of origin. Studies in animals by Borek *et al.* [8, 9] demonstrated elevated urinary levels of catabolic methylated tRNA derivatives. Additional investigations by Borek *et al.* [10] revealed that tRNA from neoplastic tissue has a much more rapid turnover rate than the tRNA from the corresponding normal tissue. Experimental evidence shows that methylation of tRNA occurs only after synthesis of the intact macromolecule. No kinases have been found that will reintroduce the nucleosides into tRNA. Consequently they are excreted following the metabolic degradation of tRNA. These urinary products include specific methylated nucleosides and pseudouridine (ψ) [11]. Studies of the metabolic fate of these compounds have been restricted to ψ. Two separate investigations have shown that ψ is not catabolized but excreted in urine directly as the intact molecule [12, 13]. Recently we developed a sensitive, specific and rapid method of analysis for urinary nucleosides utilizing reversed-phase high-performance liquid chromatography [14] following concentration by means of a boronate gel.

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Small cell carcinoma of the lung (SCC) is highly sensitive to chemotherapy and/or radiotherapy. Long-term survival occurs primarily for those patients who have a complete response following treatment, although otherwise the disease remains invariably fatal. A characteristic of SCC is its tendency towards early and distant metastases, estimated to occur in 70% of patients at the time of diagnosis [15]. This typical behavior of SCC causes problems in accurately defining tumor burden and in estimating extent of disease. Thus difficulties are frequently encountered not only at initial staging but in correctly quantitating response and assessing sites of recurrent or metastatic disease. To define these parameters better, to determine disease status more accurately and, hopefully, to aid in the definitive management of SCC, we have established a program in biomarkers for patients with SCC. The evaluation of the excretion products of tRNA as biomarkers is an integral part of this program. Five different modified nucleosides have been included: ψ, 1-methyladenosine (m^1A), 1-methylinosine (m^1I), 2-methylguanosine (m^2G) and N^2,N^2- dimethylguanosine (m^2_2G), and these were studied singly and in combination. Relationships between pretreatment levels, initial stage of disease and subsequent survival have been examined. Following treatment, comparisons have been made between serial nucleoside levels and tumor response and eventual progression. Because prior studies [16] have demonstrated the potential value of CEA as a biomarker for SCC, specific comparisons with CEA have also been made.

MATERIALS AND METHODS

Patient selection and therapy

Patients with SCC were entered into the study at The Johns Hopkins Oncology Center. The diagnostic work-up and laboratory and clinical studies carried out prior to initial therapy have been published [16–18]. Following the preliminary evaluation, patients were staged as having limited or extensive disease. Limited disease was defined as disease confined to one hemithorax, including ipsilateral hilar and mediastinal nodes. Extensive disease was defined as disease beyond the hemithorax; patients with ipsilateral supraclavicular nodes and/or pleural effusion, regardless of cytological findings, were classified as extensive stage. For those patients with extensive disease, metastatic sites were determined to include lymph nodes, bone, liver, bone marrow, central nervous system, soft tissue, pleura, heterolateral lung, homolateral lung and superior vena caval syndrome. Patients with active infection were excluded. Only patients with adequate hepatic, renal or hematological status were included.

All patients were entered onto therapeutic protocol studies which have been described previously [17, 18]. Assessment of response was made four weeks after each course of chemotherapy. Complete response (CR) was defined as the complete disappearance of all measurable lesions. Partial response (PR) was defined as a decrease of >50% in the product of the 2 longest perpendicular diameters of measurable lesions. Progressive disease (PD) was defined as an increase of >25% over the original measurements of the product of the two longest perpendicular diameters of measurable lesions and/or the occurrence of new lesions. Duration of response and survival were calculated from the first day of chemotherapy.

Sample preparation and analysis

A urine specimen for analysis, without preservative, was obtained prior to initial therapy and at approximately monthly intervals thereafter immediately before the next course of chemotherapy. Aliquot samples of each specimen were frozen in coded plastic vials and maintained at a temperature of -50°C until analysis.

Analysis was carried out by the method of Gehrke *et al.* [14] using reversed-phase high-performance liquid chromatography (HPLC) specifically developed for quantitative measurement of tRNA-modified nucleosides in biological samples. An initial isolation of the ribonucleosides with an affinity gel containing an immobilized phenylboronic acid was used to improve selectivity and sensitivity. By this method response for all nucleosides is linear from 0.1 to 50 nmol injected and good quantitation is obtained with 25 μl or less of sample placed on the HPLC column. Excellent precision of analysis for urinary nucleosides has been achieved for within and between run samples. Analytical details as to precision, recovery, chromatographic methods, minimum detection limit, retention time, relative molar response, a sample clean-up, stability of the nucleosides, boronate gel capacity and application for analysis of urine from normal subjects and patients with cancer have been published [14]. In addition, we have conducted detailed studies in the use of creatinine as a basis for ribonucleoside quantitation in urine [4]. Consequently, all analyses have been reported in nmol of nucleosides/μmol of creatinine. Normal control ratios were determined for subjects free of known diseases of the same age range as the patients in this study. CEA was determined in plasma by the Hansen method [19]. Because all the patients with SCC were cigarette smokers, a

level of 5.0 ng/ml was used as the upper limit of normal [20, 21].

Correlation with clinical parameters

The prognostic value of pretreatment levels of ribonucleosides was evaluated with respect to survival curves generated by the method of Kaplan and Meier [22]. Differences in survival between groups were compared using the generalized Wilcoxon test [23]. To examine the overall relationships between the 5 nucleosides under evaluation and disease parameters the actual measurements of nucleoside levels were normalized by the mean levels for normal controls and a composite score for the normalized levels of 5 nucleosides was determined and used to assess disease parameters.

RESULTS

Analytical characteristics

Representative HPLC chromatograms used for quantitation of results are presented in Figs 1 and 2. Figure 1 is a standard chromatogram showing the resolution of more than 30 major and modified nucleosides. Figure 2 is of a pooled urine and is representative of the HPLC method for analysis of urinary nucleosides.

Patient distribution

A total of 28 patients had urinary nucleosides determined prior to any therapy. By staging, 11 (39%) of these patients had limited disease while 17 (61%) had extensive disease. Pretreatment CEA levels were also determined for these patients.

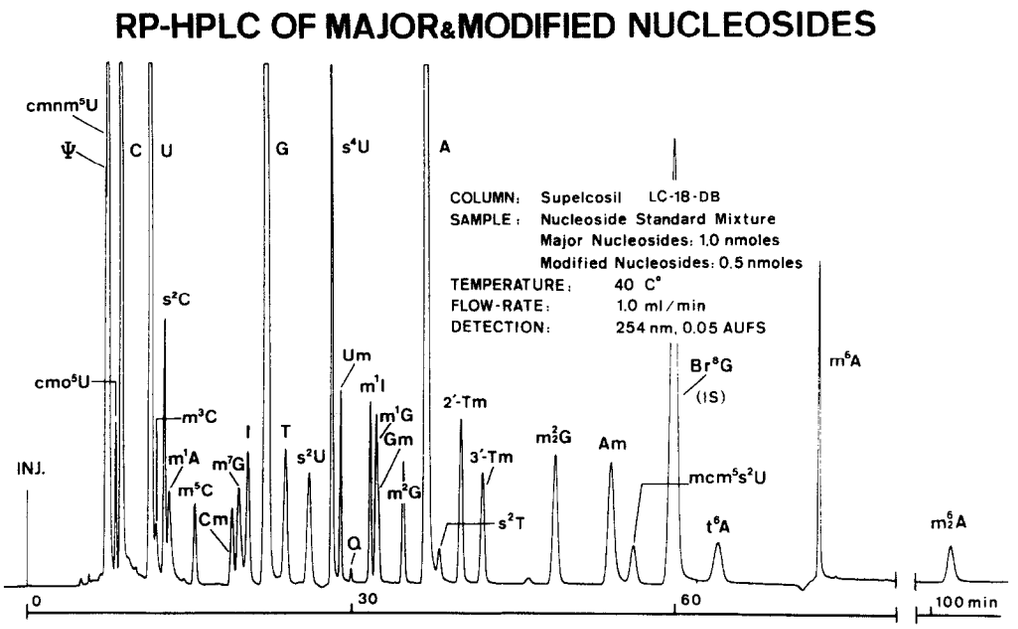

Fig. 1. HPLC chromatogram for standard compounds. Conditions are as indicated.

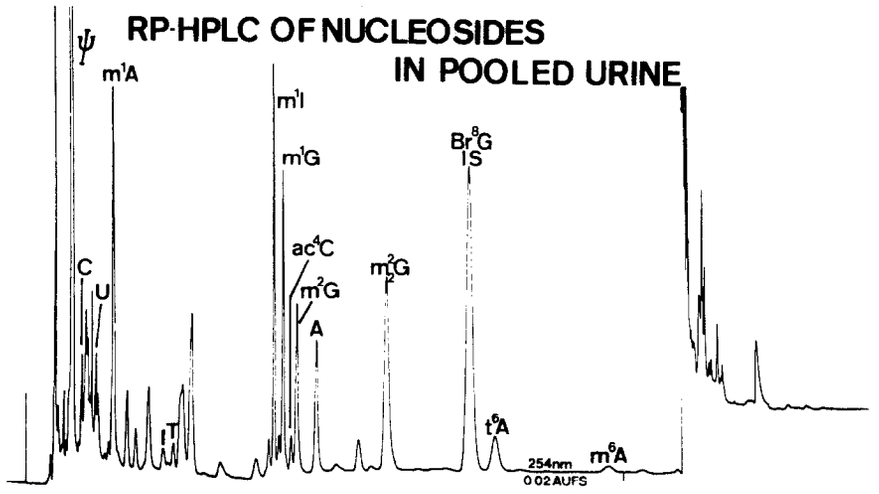

Fig. 2. HPLC chromatogram from a pooled urine sample after boronate gel isolation of the ribonucleosides.

Additional serial measurements for urinary nucleosides and CEA were made for 13 patients after one or two courses of therapy. Another 10 patients were admitted to the study but did not have pretreatment biomarker determinations. These patients were followed sequentially during the course of their disease. Both urinary nucleosides and plasma CEA were analyzed at each time interval.

A nucleoside value greater than two standard deviations above the normal mean is considered elevated. Based on stage of disease prior to therapy, the frequency of elevation of the 5 nucleosides measured in pretreatment urine samples correlated with stage of disease for the 28 patients (Table 1). A higher proportion of elevated values for all 5 of the nucleosides was found for the patients with extensive disease (Table 1, Fig. 3). Of the total determinations, 18/55 (33%) were elevated for the 11 patients with limited disease and 69/85 (81%) for the 17 patients with extensive disease. m^1A was the least frequently elevated when both limited and extensive disease was considered. One or more nucleosides were elevated for 27 of the 28 patients (96%). Variation in frequency of elevation appears to occur with different metastatic sites, as suggested by the results for m^1I, with only 2/11 or 18% elevated for limited disease patients compared fo 16/17 or 94% for those with extensive disease.

*Table 1. Pretreatment urinary nucleoside levels (nmol/μmol creatinine) for patients with SCC**

Nucleoside	Normal controls $\bar{x} + 2\sigma$	Pretreatment: stage of disease Limited $\bar{x}$ (range) median	Extensive $\bar{x}$ (range) median
ψ	24.0	30.9 (21–47) 28.0	46.0 (26–148) 41
m^1A	1.7	1.7 (0.9–3.6) 1.8	3.5 (1.4–10.2) 2.7
m^1I	1.2	1.2 (0.2–2.4) 1.3	3.2 (1.3–9.9) 2.6
m_2^2G	1.3	1.8 (1.2–4.5) 1.6	3.4 (1.3–10.0) 2.3
m^2G	0.35	0.52 (0.10–0.75) 0.50	1.43 (0.30–2.40) 1.00

*Twenty-eight patients: 11 with limited and 17 with extensive disease.

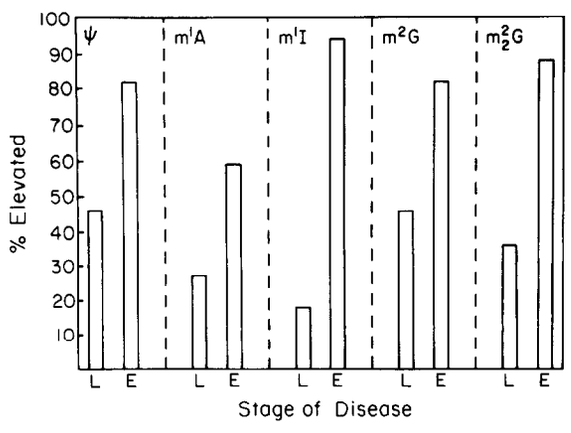

Fig. 3. Frequency of elevation for 5 individual urinary ribonucleosides based on pretreatment staging for patients with SCC: 11 with limited and 17 with extensive disease.

Frequency of nucleoside elevation in pretreatment samples and length of survival appear to be indirectly proportional. Median survival by the plot of Kaplan and Meier [22] was determined using a discriminant of 0–2 nucleosides elevated compared to 3–5 elevated (Fig. 4). A significant difference ($P = 0.004$) is demonstrated between the two groups, with a median survival of 24 months for the patients with 0–2 elevated compared to 10 months for those patients with 3–5 elevated. When examined similarly but using 0–3 compared to 4 or 5 elevated the difference in median survival is less, being 19 months for the former and 11 months for the latter ($P = 0.023$).

It should also be noted (Fig 4) that the pretreatment measurements of nucleosides were significantly related and we were able to assess the extent of disease in which only 1 of the 11 patients (9%) with 0–2 nucleosides elevated had extensive disease, whereas 16 of the 17 patients (94%) with 3–5 nucleosides elevated had extensive disease ($P = 0.001$ by the Fisher's exact test).

The composite score was derived in order to give a single value representing the summation of all 5 nucleosides determined for any point in time during the course of disease. Within the composite score each nucleoside is equally weighted by normalizing the levels in the experimental samples utilizing those respective factors calculated to convert the normal control mean level for each nucleoside to a value of one. Thus the composite score for the normal control mean values for 5 nucleosides is 5.0 and for the upper limit, i.e. 2 standard deviations above the normal mean for the total of each one of normal control values, is 7.0. It should be understood, however, that this latter number does not represent the value above which any individual score must lie to be abnormally high. An actual value for a composite score could be less and still be abnormal for any individual patient, depending on the number of nucleosides less than five which might be increased and their respective degree of elevation. The importance of a

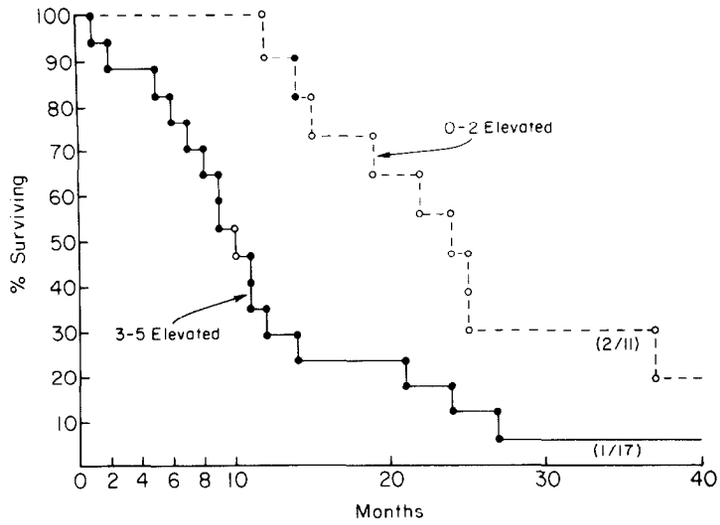

Fig. 4. Survival of 28 patients with pretreatment ribonucleosides separated based on 0–2 elevated values or >3 elevated.------ 0–2 elevated; —— 3–5 elevated; O limited disease patients; ● extensive disease patients.

composite score, or similar mathematical concept, resides primarily in the potential usefulness of such a scheme following disease response characteristics.

In Fig. 5 the composite score for each one of the pretreatment levels for the 28 patients with SCC is plotted against the corresponding CEA values. The individual points are further defined indicating the number of individual nucleosides elevated respectively and the relationship to stage of disease. The results demonstrate further that a nucleoside composite score appears directly proportional to increasing extent of disease or tumor burden. This is in contrast to the CEA results, which show a general tendency toward correlation with increasing tumor burden but with distinct variations. For example, 15 of the 28 (54%) CEA levels fall below 5 ng/ml, with 7 of these 15 (47%) representing patients with extensive disease. Similar results for CEA have been reported previously [16].

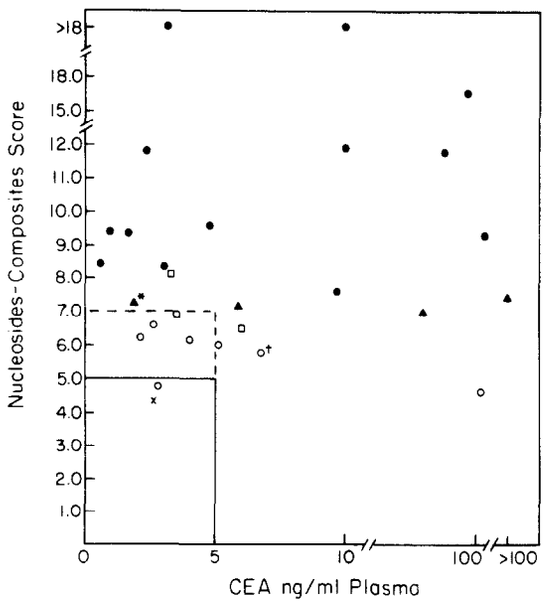

*Fig. 5. Pretreatment individual nucleoside composite scores for 28 patients plotted against their respective pretreatment CEA levels. Frequency of elevated individual nucleosides indicated: ×, 0 elevated; O, 1 elevated; □, 2 elevated; △, 3 elevated; ●, 4 or more elevated; *only patient with limited disease with 3 or more elevated; †only patient with one or less elevated with extensive disease. The linear regression was determined; NUC-CS = 6.78 + 0.425 CEA, r = 0.652 (P<0.001).*

The relationship between changes in composite score for the nucleosides and changes in tumor burden associated with response to therapy is shown in Fig. 6 for those patients with extensive disease who had pretreatment and serial urine determinations. Similar curves (Fig. 7) are presented for patients who entered the study sometime after initial treatment during the course of disease. The latter primarily demonstrate changes with disease progression associated with resistance to therapy. For comparison, CEA levels are plotted for the same serial time points. Although discordant results are noted, the general pattern of change in values corresponds to change in tumor burden. The nucleoside composite score and CEA time curves tend to be similar, but occasional differences can be noted.

DISCUSSION

Neoplastic tumor tissue has invariably been shown to increase tRNA methyltransferase activity compared to the corresponding normal tissue of origin [8]. However, the primary reason for elevation of urinary degradation products of tRNA for patients with cancer is probably the more rapid turn-over rate for tumor tRNA when

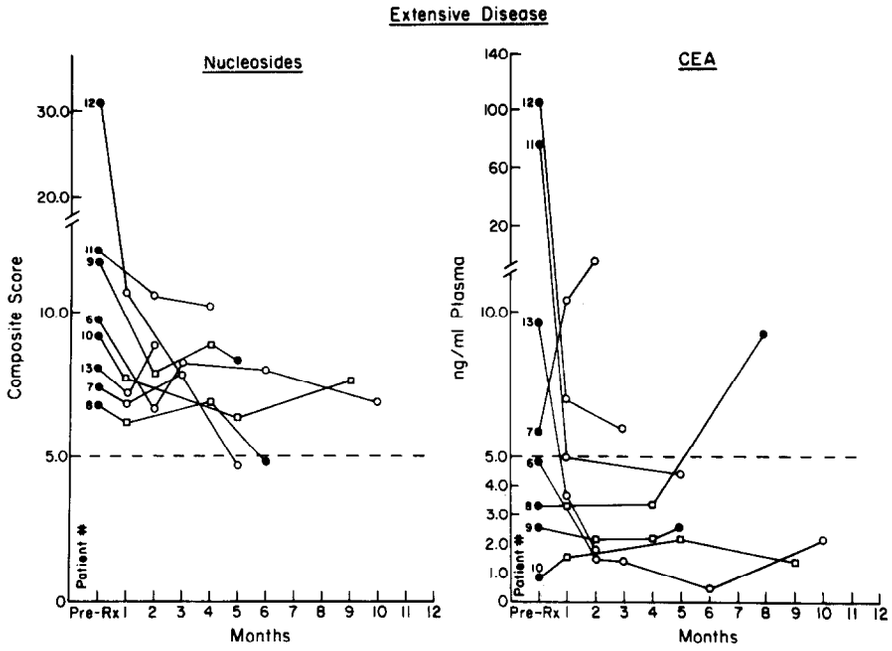

Fig. 6. Serial composite scores and CEA levels for patients with extensive disease during therapy.

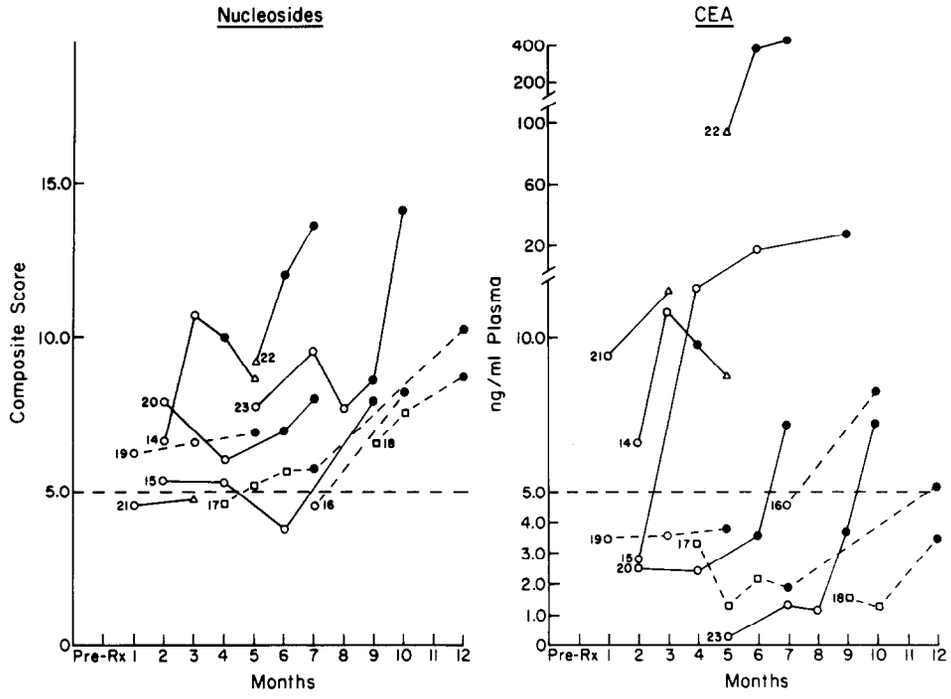

Fig. 7. Serial composite scores and CEA levels for patients who entered the study after therapy had been initiated. -----Pretreatment limited disease; —— pretreatment extensive disease.

compared to its normal tissue counterpart [10]. Further evidence suggests that the frequency and degree of elevation of individual modified tRNA nucleosides excreted in the urine is probably related to kinetic growth parameters of the tumors involved. This has been found in studies of patients with Burkitt's lymphoma [3], a tumor with a high proliferative growth fraction and cell death per unit of time and, in contrast to breast cancer [7], a tumor with a higher proportion of cells in G_0 phase. Although studies to evaluate the ability of tRNA nucleosides as an adjunctive means for following tumor response and progression during therapy have been limited, preliminary data indicate that these materials may be of most value in more rapidly growing and/or chemotherapeutically sensitive tumors [3; Waalkes *et al.*, unpublished observations]. Prior results [24] suggested that ψ levels parallel changes in disease status for patients with chronic

lymphocytic leukemia. More recently preliminary findings show that modified tRNA breakdown products are excreted in greatest amounts for those patients with non-Hodgkin's lymphoma who have stage III or IV disease (Waalkes *et al.*, unpublished observations). Because of these tumor-related properties and the rapid growth and response characteristics of SCC, studies were undertaken to define the possible ability of tRNA degradation products to act as biomarkers for this disease. Of particular advantage, the recently developed analytical method utilized in this study permits, by one chromatographic run, the analysis and subsequent direct comparison of a large number of modified nucleosides in the same urine sample.

The results of this study depict a direct correlation between frequency and degree of elevation for each one of the five individual nucleosides with stage or extent of primary disease. Although occasional exceptions are noted, in general a direct relationship occurs between nucleoside excretion and tumor burden. This is based in part on the assumption that in most instances stage of disease and tumor burden are directly related. With a greater number of metastatic sites, the frequency and degree of elevation of the nucleosides increased. As shown in Fig. 3, a significant relationship is noted between the number of nucleosides elevated in pretreatment urines and stage of disease. Similarly, a high degree of correlation exists between the number of nucleosides elevated per individual patient and length of survival (Fig. 4).

The use of a composite score as a representative number for the summation of all 5 nucleosides provides the advantage of a single number to indicate disease response at each serial time point during the course of disease. Although giving an equal weight to each nucleoside measurement under all changing clinical situations may be in error, nevertheless, as shown, general agreement was found when the composite score was equated against clinical response parameters. More precise, mathematical approaches await the study of a greater number of patients to gain a better appreciation of the relative values of each nucleoside determination in contrast to clinical parameters. In a prior preliminary study [25] we evaluated two individual nucleosides for their respective ability to monitor the disease course for patients with SCC and found an overall estimated concordance of 75%. A greater frequency of discordant values were noted for patients with limited disease and lower nucleoside values. Similar results had also been seen for CEA [16]. However, other tentative causes for discordant results should be considered. Undoubtedly a broad range of possible error lies in the common difficulty associated with assessing tumor response by clinical measurement. Particularly for patients with SCC, the estimation of total tumor burden or change with therapy can be difficult because the clinical assessment of objective disease parameters may be relatively gross due to the widely metastatic nature of the disease. This may explain some of the apparent disagreements between biomarker measurements and clinical estimation of tumor status. Other reasons for discordant results are unclear. The heterogeneity of tumors between patients, and for the same patient between metastic sites [26], could be important factors. The possibility exists that therapy and associated biochemical changes within responding or progressing tumors may be related to alterations in the activity of individual isoaccepting tRNA methyltransferase enzymes. Prior *in vitro* studies [27] with commonly used chemotherapeutic agents did not show an affect on total tumor tRNA methyltransferase activity unless protein synthesis was also inhibited. Individual tRNA methyltransferase enzymes, however, were not studied.

In summary, the pretreatment levels for modified degradation products of tRNA excreted as nucleosides in the urine of patients with SCC are often elevated and correlate with stage of disease and tumor burden. Based on a discriminant using 0–2 nucleosides elevated or 3–5 in pretreatment samples, highly significant relationships are found directly with stage of disease and tumor burden and with survival. Using a composite score for summation of nucleoside analyses during the course of disease showed a general agreement with clinical parameters of response or progression. A major advantage for using the nucleosides as possible biomarkers lies in the HPLC method. All of them can accurately and quickly be measured per individual urine sample in one HPLC run. Although further studies are needed, the use of nucleosides in combination with other biomarkers, e.g. CEA, may eventually have potential application as an adjunct to the clinical assessment for each patient.

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