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Cancer-specific Genomic Instability in Bronchial Lavage: A Molecular Tool for Lung Cancer Detection[1]

Triantafillos Liloglou, Paul Maloney, George Xinarianos, Melanie Hulbert, Martin J. Walshaw, John R. Gosney, Lesley Turnbull, and John K. Field[2]

Roy Castle International Centre for Lung Cancer Research, Molecular Oncology Unit, Liverpool L3 9TA [T. L., P. M., G. X., M. H., J. K. F.]; Departments of Clinical Dental Sciences L69 3BX [T. L., P. M., G. X., M. H., J. K. F.] and Pathology, Liverpool L69 3GA [J. R. G., L. T.], University of Liverpool; and Cardiothoracic Centre, Liverpool L14 3PE 4 [M. J. W.], United Kingdom

ABSTRACT

We examined genomic instability in DNA from 80 bronchial lavage samples from patients with lung cancer and individuals with no malignant lung disease. We used a multiplex assay of eight fluorescent-tagged microsatellite markers that have a very high incidence of allelic imbalance in lung tumors. When genomic instability at individual loci was analyzed statistically against diagnosis, markers *D3S1289* ($P = 0.033$), *D3S1300* ($P = 0.001$), *D13S171* ($P = 0.009$), and *D17S2179E* ($P = 0.017$) demonstrated significantly higher frequency of instability in bronchial lavage specimens from lung cancer cases than those with nonmalignant conditions. In contrast, markers *D9S157, D9S161, D13S153,* and *D5S644* demonstrated lower specificity ($P > 0.05$) for lung tumors. These results suggest that genomic instability in some loci may be related to high proliferation rates but not necessarily to cell commitment to malignancy. When genomic instability was scored with only the four cancer-specific markers, the assay produced a sensitivity of 73.9% and a specificity of 76.5%. On combining the results from the cytological examination and the molecular assay, the sensitivity reached 82.6%. These results indicate that in our efforts to investigate genomic instability as a potential marker for the early detection of lung cancer, we need to identify cancer-specific genomic instability markers. This paper has shown that these first four markers may be considered to form an individual set of cancer-specific genomic instability markers.

INTRODUCTION

Genomic instability is the most common molecular abnormality in human tumor cells (1, 2). One form of genomic instability is allelic imbalance or LOH[3] that reflects chromosomal instability, *i.e.,* epigenetic changes such as aneuploidy, polyploidy, losses, and amplifications of chromosomal regions. The other form of genomic instability is MSI, also referred to as microsatellite alterations or replication errors, representing replication and DNA repair infidelity. The high incidence of genomic instability in lung tumors has been well established (3–7), and in some cases it has been associated to prognosis (8–10). We have recently demonstrated genetic alterations in 97.6% of lung tumors examined by a panel of 12 microsatellite markers selected at specific locations (11). We have also calculated the threshold of LOH detection to 23% by assessing the interassay variation.

Lung cancer is the most common cause of neoplasia-related death worldwide. Moreover, it usually has very poor prognosis with a ≤6% 5-year survival (12). One of the reasons for this low survival is that cancer is most often diagnosed when it is beyond effective treatment. Thus, there is an increasing demand for new early lung cancer detection tools (13, 14). Lung cancer develops through a multistage process of steps with increasing genomic instability. Genetic alterations have been detected in preneoplastic lung (15–18) and esophageal (19, 20) lesions as well as in bronchial tissue from smokers with no evidence of lung malignancy (21, 22). DNA aberrations precede morphological transformation (23) and thus are favorable markers and potential tools for the identification of individuals at high risk for developing lung cancer. It has been previously shown that genomic instability can be detected in bronchial lavage and sputum, and this may be one of the ways forward to assist in early diagnosis of lung cancer (24–27). We have demonstrated genomic instability in BL from a number of individuals with no clinical evidence of lung cancer, posing a question about exclusive occurrence of genomic instability in cancer (25). This observation was also supported by reports of genomic instability in nonmalignant diseases (28–34).

The technological advantages of fluorescence PCR-based assays provide the ability to detect DNA changes from minute amounts of starting material in multiplex reactions (35). Furthermore, automated analysis on sequencers/genetic analyzers not only increases throughput but also reduces operator errors during analysis. In the present study, we selected a panel of eight microsatellite markers that were found to carry LOH in >95% of lung tumors (11) and used them to validate genomic instability as a potential detection tool in individuals with and without lung clinical lung cancer.

MATERIALS AND METHODS

Patients and Clinical Samples. We have collected 80 BL and corresponding blood samples from individuals with suspected lung cancer who have been referred to the Cardiothoracic Center in Liverpool. Patients were selected on the basis of an adequate cytology preparation, blood sample availability, as well as initial clinical diagnosis (*i.e.,* two groups, lung cancer and nonmalignant diseases) Each patient underwent a full clinical workup for lung cancer including a chest X-ray, spirometry, and bronchoscopy. Bronchial lavage specimens were obtained from all of these patients; the choice of site was based on bronchoscopic findings within the large airways, where approximately 50 ml of saline were introduced via the bronchoscope and then aspirated. The age of the patients selected ranged between 38 and 89 (average, 65). Twenty-nine of the individuals were female, and 51 were male. The initial pathological/clinical diagnosis was: 13 adenocarcinomas; 22 squamous cell carcinomas (squamous cell carcinoma of the lung); 10 small cell carcinomas (small cell lung carcinoma); 10 asthma; 3 bronchial hyper reactivity; 16 chest infections (*e.g.,* pneumonia); and 6 COPD patients. Follow-up information revealed that one of the patients with an initial COPD diagnosis at the time that BL was taken was diagnosed with a lung tumor (small cell lung carcinoma) 4 months later (patient B083). Smoking data were available for 69 individuals (51 current smokers, 13 former smokers, and 5 nonsmokers). The total smoking exposure was calculated in pack-years = [(age at presentation − age started − years stopped) × (cigarettes/day)]. A differential cell count was undertaken for all bronchial lavage samples reported as "no malignant cells seen" and the lung cancer patients with genomic instability. The epithelial cells present varied between samples (25–90%).

DNA Extraction and PCR. DNA from blood was extracted with the Qiamp96 extraction kit (Qiagen, Ltd., West Sussex, United Kingdom) following the supplier's protocol. BL was centrifuged for 5 min at 2000 × *g,* and the

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[2] To whom requests for reprints should be addressed, at Roy Castle International Centre for Lung Cancer Research, Molecular Oncology Unit, 200 London Road, Liverpool L3 9TA, United Kingdom. E-mail: J.K.Field@liv.ac.uk.

[3] The abbreviations used are: BL, bronchial lavage; LOH, loss of heterozygosity; MSI, microsatellite instability; CSGI, cancer-specific genomic instability; COPD, chronic obstructive pulmonary disease.

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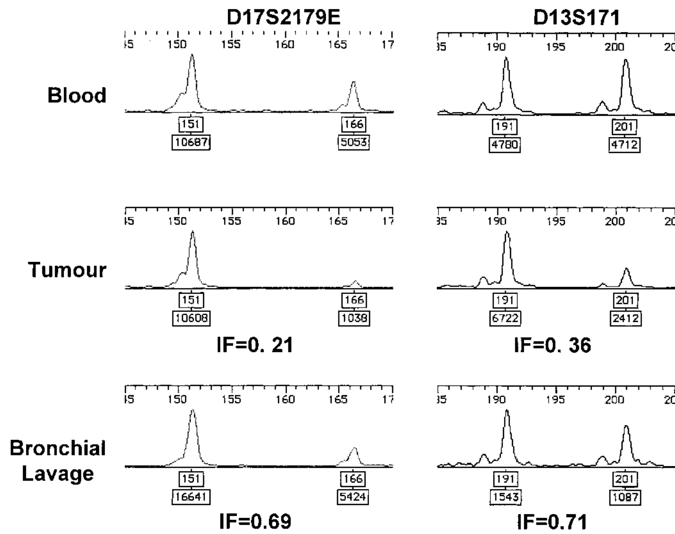

Fig. 1. Examples of LOH in tumors and corresponding bronchial lavage samples. The imbalance factor (*IF*) increases in the BL due to the presence of normal contaminating DNA; however, LOH is still detectable because the imbalance factor is below the detection threshold (0.77).

resulting pellet, containing variable amount of mucus, was processed using the DNAeasy 96 kit (Qiagen, Ltd.).

Primers for the microsatellite loci *D3S1300, D3S1289, D5S644, D9S161, D9S157, D13S153,* and *D13S171* were selected from the LMS High Density Panel Set (PE Applied Biosystems, Warrington, United Kingdom). The primers for *D17S2179E* (p53 intron 1) were designed using OLIGO software to amplify in the same conditions with the LMS panel: forward, 5′-AGTA-AGCGGAGATAGTGCCA-3′; and reverse 5′-GCACTGACAAAACATC-CCCT-3′. The 10-μl multiplex PCR mixture contained 1× Gold Buffer (Applied Biosystems), 2.5 mM $MgCl_2$, 500 μM dNTPs and 0.75–1.0 μM concentrations of each primer pair, 0.75 unit Amplitaq Gold (Applied Biosystems), and 2–3 μl of the extracted DNA. The thermal profile was 95°C for 12 min followed by 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. A 30-min final extension step was included at the end to ensure maximum nontemplate–A addition and thus eliminate split peaks. Two μl of the PCR product were mixed with 3 μl of loading buffer (formamide:dextran blue:EDTA, 5:1:1; ROX 350 size standard). The mixture was denatured at 95°C for 5 min, chilled on ice, and loaded on a 6% denaturing polyacrylamide gel on a 377 ABI PRISM automatic sequencer. The gel image was analyzed using the Genescan and Genotyper software (Applied Biosystems).

LOH and MSI Scoring. MSI was scored when a novel allele was present in the BL. LOH was scored as previously described (11). Briefly, a 0.23 threshold has been established based on a 99% reference range (=3× SD) of 1126 allele ratios coming from normal sample multiple repeats, and thus we assess the interassay variability. Such a calculation approach does not rely on the source of DNA, because it scores as positive the values falling outside a calculated region of normal variation of the reaction. BL:blood ratios were scored as: negative if $0.77 < A_x$; LOH if $A_x \leq 0.77$ or $A_x \geq 1.23$. To avoid false positives due to PCR artifacts, all of the samples were assayed twice; when inconsistencies in values around the boundaries were observed, a third repeat was performed.

RESULTS

We initially assessed the value of the information from a panel of eight markers. Heterozygosity levels in the examined markers ranged between 0.58 and 0.81. Of the 80 samples, 67 had ≥4 informative markers with an average heterozygosity of 0.68 (equivalent of 5–6 informative markers per sample). LOH/MSI in at least one of the 8 examined markers was detected in 40 of 46 (86.9%) lung cancer cases (36 LOH, 4 LOH + MSI). In three cases, DNA available from tumor tissue was also analyzed and revealed loss of the same allele in the tumor and the corresponding BL sample (Fig. 1). Also, one patient (B083), who was initially diagnosed with nonmalignant disease but 4 months later was diagnosed with a lung tumor had LOH at *D3S1289* and *D9S161* and MSI in *D9S157.* However, LOH/MSI was also detected in 26 of 34 (76.5%) individuals with no malignant disease (24 LOH, 2 LOH + MSI). All LOH/MSI results have been confirmed by repeating a separate PCR on at least two occasions. The analytical results for all markers are given per diagnosis group in Table 1.

On analyzing the LOH/MSI of individual markers, LOH/MSI results with regard to diagnosis, we found that four markers demonstrated high specificity for lung cancer cases (*D3S1289,* $P = 0.033$; *D3S1300,* $P = 0.001$; *D13S171,* $P = 0.009$; *D17S2179E,* $P = 0.017$) whereas the remaining markers (*D9S157, D9S161, D13S153,* and *D5S644*) demonstrated lower specificity for lung cancer exhibiting LOH/MSI in numerous nonmalignant disease samples as well (Fig. 2). The panel of the four CSGI markers was analyzed, 34 of 46 (31 LOH, 1 MSI, 2 LOH + MSI) lung cancer cases (sensitivity, 73.9%) and 8/34 (7 LOH, 1 MSI) nonmalignant cases scored positive (specificity, 76.5%; $P = 3.3 \times 10^{-5}$) (Fig. 3). CSGI detection sensitivity was higher than that of cytology ($P = 0.04$) (Table 2). On combining cytology and CSGI data, the sensitivity increases to 82.6% (comparison with cytology alone, $P = 0.007$).

Another analytical approach undertaken to increase cancer-specificity scoring was to include in the positive group only individuals with LOH/MSI at ≥2 loci. This analysis revealed 35 of 46 (76.1%) of lung cancer cases positive, whereas 12 of 34 (35.3%) nonmalignant cases ($P = 2.7 \times 10^{-4}$). This approach provides a gain of only 2.2% in sensitivity over the previous CSGI markers approach, but an 11.8% drop in specificity was found. Thus, the combination of cytological examination with the CSGI markers provides the highest levels of sensitivity and specificity (Table 2).

No association was demonstrated between CSGI alterations and age, gender, and smoking. Regarding the latter, only five nonsmokers were included in this study; therefore, no valid statistics could be applied to compare smokers with nonsmokers. No difference was

Table 1 *Genetic alterations (LOH/MSI) in BL specimens from patients with and without lung cancer*

	D3S1300		D3S1289		D5S644		D9S161		D9S157		D13S153		D13S171		D17S2179E	
Diagnosis	+[a]	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
AdenoCa[b]	4	5	2	8	8	4	1	8	4	5	5	8	4	6	4	3
SqCCL	7	12	2	4	6	3	4	10	5	8	10	8	7	8	4	1
SCLC	3	4	9	3	5	2	5	3	5	1	3	2	3	3	6	10
Total carcinomas	14	21	13	15	19	9	10	21	10	14	18	18	14	17	14	14
Asthma	1	8	1	8	5	3	1	8	2	6	1	6	1	5	1	5
COPD	0	5	0	4	0	5	1	4	0	4	3	2	0	4	0	2
Chest infection	1	13	4	7	4	2	0	11	3	6	9	6	2	10	2	8
BHR	0	3	0	2	0	2	1	0	0	2	1	1	0	2	0	1
Total nonmalignant	2	29	5	21	9	12	3	23	5	18	14	15	3	21	3	16

[a] Genetic alterations: +, LOH or MSI; −, no LOH/MSI. The table includes only informative (heterozygous) samples for each marker.
[b] AdenoCa, adenocarcinoma; SqCCL, squamous cell carcinoma; SCLC, small cell lung carcinoma; BHR, bronchial hyperreactivity.

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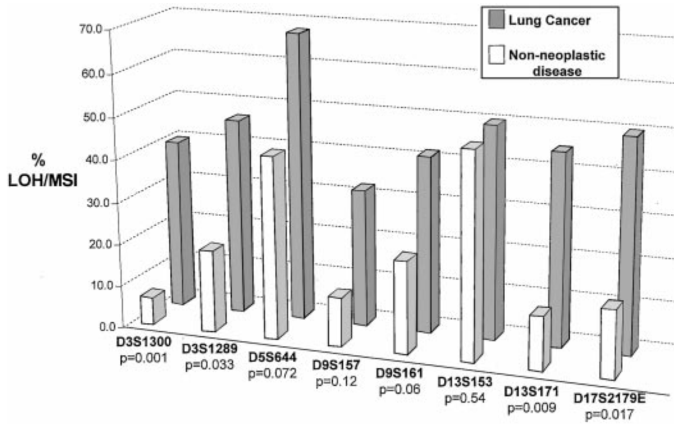

Fig. 2. Histogram demonstrating the frequency of LOH/MSI in the examined markers in BL from lung cancer patients and individuals with no evidence of lung neoplasia. *P*s (Fisher's exact test) indicate the specificity of each marker for lung cancer.

observed in CSGI detection frequency among current and former smokers, different daily tobacco exposure (cigarettes/day), or overall tobacco exposure (pack-years) groups. In addition, in cancer cases no association was found between CSGI alterations and tumor histological type, size (T stage), and nodal metastasis. Interestingly, of the eight individuals with non malignant lung disease found with CSGI alterations, four had asthma, and four had chest infections.

DISCUSSION

Genomic instability is present in virtually all tumors, making such a molecular abnormality a favorable biomarker for detection of cancer by examining DNA from body fluids (24–27, 36–38). Thus, one can detect cancer cells by tracing the DNA damage that they carry. The high frequency of this genomic aberration in lung neoplasia as well as recent fluorescent PCR based methods and automation provide a way of attaining this objective. Because genetic damage precedes morphological transformation, molecular assays should be capable of detecting genetically abnormal cells that escape cytological examination. Detection of genetic alterations in bronchial lavage and sputum has been previously reported (24–27), demonstrating the value of this technique in lung cancer detection. However, the sensitivity and specificity of such assays require serious improvement before becoming diagnostic tools.

The most frequent LOH regions may differ among various tumor types (39) and possibly sites; thus, the most appropriate panel of chromosomal loci must be determined for a given tumor type. In this study, we have selected eight microsatellite markers with a high heterozygosity that have shown LOH/MSI in >95% of lung tumors (11). The PCR assay was optimized in a multiplex reaction to increase throughput and also to reduce the amount of DNA required. In addition, the threshold of LOH detection was calculated to 0.23 by assessing the interassay variation rather than arbitrarily selected, thus, LOH is scored if the target:reference allele ratio lies outside a 99% reference range calculated on 1126 values from normal DNAs (11). This increases sensitivity without increasing the false positives.

When samples were scored "positive" on the basis of at least one marker carrying LOH/MSI, a specificity of 23.5% was observed. Further analysis revealed two groups of markers: cancer-specific (*D3S1289, D3S1300, D13S171,* and *D17S2179E*) and low specificity markers (*D9S157, D9S161, D13S153,* and *D5S644*). We have previously demonstrated microsatellite instability in the bronchial lavage of individuals with no evidence of lung cancer and in particular in individuals with chronic conditions such as fibrosing alveolitis, rheumatoid arthritis, and cardiac problems (25), posing the question whether these individuals are at higher risk of developing lung cancer or if the detected genetic alteration was merely indicative of a non-neoplastic inflammatory process. Further support for this argument comes from reports on genomic instability in nonneoplastic diseases such as COPD (28–29), pulmonary sarcoidosis (33), rheumatoid arthritis (34), and chronic ulcerative colitis (30–32). Thus, genomic instability is not an exclusive feature of neoplasia; it may also be associated with chronic inflammatory processes and autoimmune diseases. Moreover, bronchial specimens from smokers with no evidence of lung neoplasia have been shown to carry genetic alterations (21, 22). These genetic alterations reflect chronic exposure to tobacco carcinogens but may not necessarily commit the cell to neoplasia, given that only approximately 20% of smokers will eventually de-

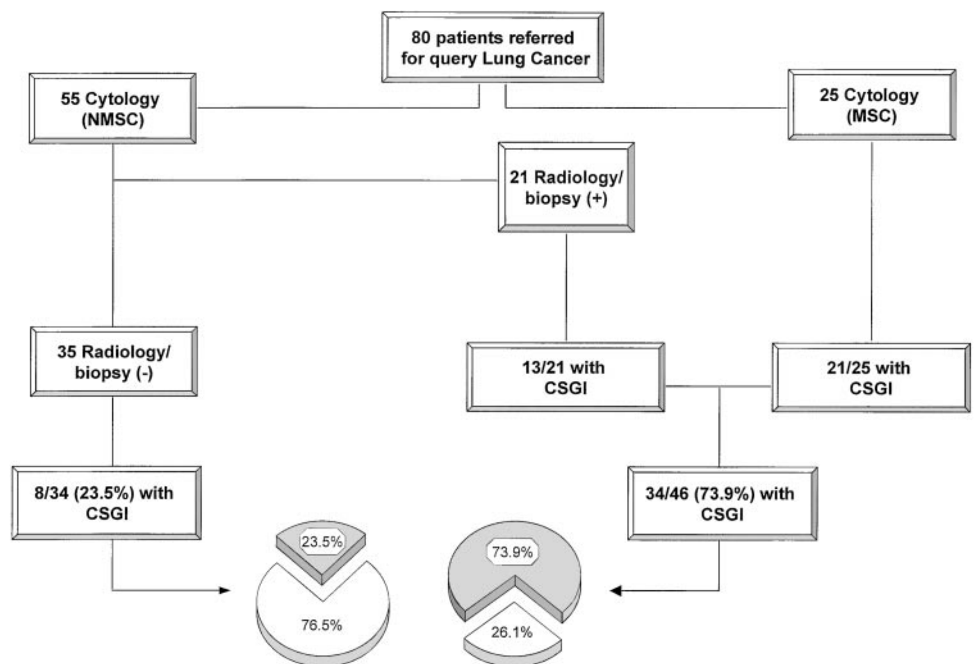

Fig. 3. Diagram demonstrating the sensitivity and specificity of CSGI in the BL of individuals with and without lung cancer and with regard to the cytological examination. *MSC*, malignant cells seen; *NMSC*, no malignant cells seen. Pie charts: *white slice,* CSGI negative; *filled slice,* CSGI positive.

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Table 2 *Sensitivity and specificity of the microsatellite analysis and cytology examination in BL from patients with and without a clinical diagnosis of lung cancer*

	Cytology		Cancer-specific markers		Cancer-specific markers + cytology	
	−	+	−	+	−	+
Lung cancer	21	25	12	34	8	38
Nonmalignant disease	34	0	26	8	26	8
Sensitivity (%)	54.3		73.9		82.6	
Specificity (%)	100		76.5		76.5	

velop lung cancer. Thus, we need to distinguish CSGI from instability indicative of nonneoplastic disorders. Indeed, our results are supportive of such a hypothesis by presenting markers associated with neoplastic lung disease and markers having no or low specificity for neoplasia. Further support comes from a study also reporting LOH in bronchial brushings from patients without lung cancer (27). In the latter study, the authors have undertaken a quantitative approach toward cancer specific scoring by introducing a "LOH score." Thus, the need to determine assays able to distinguish CSGI from noncancer-indicative genetic alterations becomes apparent.

No association was observed between genomic instability and smoking parameters. However, only five nonsmokers were included in this study, making valid statistical comparisons only among daily tobacco exposure, overall tobacco exposure, and current-former smoker status groups. The lack of any association between CSGI and smoking parameters in both the lung cancer and nonneoplastic disease groups indicates that such genetic alterations most likely occur early in the development of these lesions. This is also supported by a report demonstrating similar LOH frequencies at chromosome 8p in current and former smokers without cancer (40). In addition, the lack of association of CSGI with tumor size and nodal metastasis in the present study further suggests that these are early changes.

In this study, we considered both LOH and MSI as "positive genomic instability." However, these two genetic alterations reflect different genomic abnormalities. We were not able to assess the relative cancer-specificity of LOH in comparison with MSI as only 6 of 80 samples in total had MSI. This is most likely because the markers examined have shown infrequent MSI incidence in lung tumors (11). A number of other markers with high frequency of MSI in lung tumors have been reported (26), and further investigation is required to elucidate the relative detection impact of these two types of genetic alterations.

In the present study, we demonstrated 73.9% sensitivity and 76.5% specificity by using only the four CSGI markers. This is higher than the cytological examination sensitivity ($P = 0.04$) Moreover, when CSGI data are combined with cytological examination, the sensitivity increases to 82.6%, which is not as yet sufficient for clinical trials; however, it is based on only four markers. Additional studies are currently being undertaken to expand this set of CSGI markers. By increasing the number of CSGI markers, a higher sensitivity will be achieved. On the other hand, the set of CSGI markers had a 23.5% false positive rate. However, the individuals with nonmalignant disease in this study do not comprise a strict "control" population, because they are high risk for lung cancer individuals. A clear example of this is patient B023 who changed status 4 months after initial diagnosis as noted in the results. All eight CSGI-positive individuals with no malignant disease are currently being followed up to assess whether they are real false positives due to the method (associated with nonneoplastic disease instability) or if it is an early indication of lung cancer. The current false positive rate makes this set of four markers unlikely to be diagnostic at this time, and additional microsatellite markers must be tested to increase the cancer specificity. However, our results suggest that in conjunction with conventional clinical assays, CSGI is a useful surrogate biomarker for the detection of lung cancer. Future investigations are required to expand the current set of CSGI markers to achieve sensitivity and specificity figures that would allow progress into clinical trials.

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