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Published in:
Journal of International Medical Research

DOI:
10.1177/03000605221108924

Publication date:
2022

Document version:
Final published version

Pre-Clinical Research Reports

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
50(6) 1–10

DOI: 10.1177/03000605221108924
journals.sagepub.com/home/imr
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Natural killer cell activity as a biomarker for the diagnosis of lung cancer in high-risk patients

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Abstract

Objective: Natural killer (NK) cells play an essential role in the immune response against cancer. However, immune escape mechanisms may cause inferior NK cell activity (NKA) in patients with cancer. This prospective study examined the relationship between NKA and lung cancer in a high-risk cohort.

Methods: In a cohort study, 250 participants referred by their general practitioner for suspicion of lung cancer were included. Before clinical investigation, blood was collected into NK Vue tubes, and the level of interferon gamma after 24 hours served as a surrogate marker for NKA.

Results: Among 250 patients, 79 were diagnosed with lung cancer. No difference in NKA was found between patients with lung cancer and control participants in which lung cancer was ruled out (median 226 pg/mL vs. 450 pg/mL). However, there was a significant difference in NKA between patients with late-stage lung cancer and controls (median 161 pg/mL vs. 450 pg/mL). A linear regression model showed that NKA was not influenced by age, sex or smoking status.

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Conclusions: The significantly lower NKA in patients with late-stage lung cancer warrants further investigation combining NKA with other biomarkers and examining the potential role of NKA as a marker of disseminated disease.

Keywords
Natural killer cell, lung cancer, interferon gamma, screening, biomarker, high-risk

Date received: 28 February 2022; accepted: 6 June 2022

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, and lung cancer incidence is expected to increase.[1] The stage of lung cancer assigned at diagnosis is pivotal for patient survival,[2] emphasizing the need for early detection. Hence, there is a demand for diagnostic biomarkers, and various molecular candidates, such as circulating DNA, DNA methylation and proteins, are currently being examined.[3–5]

Evidence positions natural killer (NK) cells as important contributors to the immune response against cancer. NK cells are cytotoxic innate lymphocytes that use tightly regulated processes to kill virally-infected cells and cancer cells without prior activation or sensitization.[6] Moreover, NK cells secrete diverse cytokines, including tumor necrosis factor alpha and interferon gamma (IFNγ), that orchestrate innate and adaptive immune system processes and directly affect cancer cells.[7,8]

A large epidemiologic study with 11 years of follow-up found that low NK cell activity (NKA) was associated with increased cancer risk, suggesting an inverse relationship between the immunological response of NK cells and cancer incidence.[9] Furthermore, studies in various cancer types have shown that NK cell effector functions are suppressed by several events, including repeated exposure to tumor ligands, high expression of checkpoint ligands in the tumor microenvironment, exposure to different immune cell types and elevated concentrations of transforming growth factor beta and interleukin-10.[10–13] In lung cancer, a study performed using peripheral blood showed that NKA in these patients was inferior to that in a control cohort. In addition, a second study found that NK cells in adenocarcinoma lesions were decreased and expressed minimal amounts of granzyme B, IFNγ and CD57.[14,15] These changes, likely induced by the presence of malignancy, indicate the potential use of NKA measurements for the detection of cancer.

The primary aim of the current study was to evaluate the level of NKA in a high-risk cohort of patients referred by their general practitioner for suspicion of lung cancer. Secondary aims included the comparison of NKA levels between participants in which cancer was ruled out and patients with early-stage or late-stage lung cancer and the assessment of the potential influence of clinical factors on NKA levels.

Materials and methods

Study design and patient cohort

A high-risk cohort was consecutively recruited at the Department of Medicine, Vejle Hospital, University Hospital of

Southern Denmark, Vejle, Denmark, from February 2019 to January 2020 after being referred by their general practitioner for suspicion of lung cancer. Diagnostic workup was performed as recommended by international guidelines,[16] and lung cancer staging was according to the IASLC 8th edition.[17]

In this cohort study, the inclusion criteria were defined as 1) referred for suspicion of lung cancer, 2) age above 18 years, and 3) written and verbal informed consent. Exclusion criteria were 1) previous lung cancer, 2) other malignant diseases 5 years prior to study enrolment, except basal cell or squamous cell carcinoma of the skin and carcinoma in situ cervicis uteri, and 3) severe comorbidity causing patients to be incapable of participating in diagnostic procedures.

The reporting of this study conforms to STROBE guidelines.[18]

NKA measurement

Before diagnostic workup, blood samples were obtained, and NKA was measured in accordance with recommendations.[19] Briefly, 1 mL of whole blood was drawn into NK Vue® tubes (NKMAX Co., Ltd., Seongnam-si, South Korea) and placed at 37°C within 15 minutes of sampling. After 24 hours, the plasma level of IFNγ was measured using the NK Vue® ELISA (NKMAX). Values below the lower limit of quantification (65 pg/mL) were recorded as 32.5 pg/mL. Samples with test results above the assay's upper limit (2000 pg/mL) were diluted at 1:10 and reanalyzed. The in-house intra-assay and inter-assay coefficients of variation of the ELISA were $<10\%$ and $<12\%$, respectively, and the lower reference limit was 120 pg/mL measured in-house. A cut-off of 250 pg/mL was used to separate abnormal from normal NKA in accordance with the manufacturer's suggestion. We previously showed that IFNγ is predominantly secreted from NK cells.[20]

Statistics

Non-normal distributed values are described as the median and interquartile range (IQR). Whiskers in boxplots are the smallest value greater than Q1 – $(1.5 \times \text{IQR})$ and the greatest value smaller than Q3 + $(1.5 \times \text{IQR})$. Categorical values are presented as frequencies and percentages. The Mann–Whitney U test was used to compare continuous variables. Receiver operating characteristic (ROC) curves were analyzed to determine the optimal cut-off value using the Youden index. The risk of lung cancer at different cut-off values was assessed using odds ratio contingency analysis, with significance measured with Fisher's exact test. Multiple linear regression was performed to evaluate the influence of clinical factors on NKA. $p < 0.05$ was considered statistically significant. All statistical analyses were calculated using R (www.r-project.org), and figures were produced using the R package ggplot2.[21]

Ethics

The study was approved by the Regional Committee on Health Research Ethics in Southern Denmark (ID: S-20180052) and the Danish Data Protection Agency (ID: 18/33058). All participants provided written informed consent to participate.

Results

Clinical features

Among the 250 participants in the high-risk cohort, 79 patients were diagnosed with lung cancer, including 29 in stage I to II and 50 in stage III to IV. For the remaining 171 participants, lung cancer was ruled out, and they served as control subjects.

The descriptive characteristics of participants in the two groups are presented in Table 1. Patients with lung cancer were slightly older ($p < 0.05$), and the two groups had a similar sex distribution. A larger percentage of patients with lung cancer were ever-smokers (91%) compared with control subjects (70%).

NKA

NKA measured by the concentration of IFNγ released in the plasma was assessed across groups consisting of control subjects and patients with any-stage, early-stage (stage I–II) and late-stage (stage III–IV) lung cancer (Figure 1). There were no differences in IFNγ release between control subjects (median 450 pg/mL, IQR 130–1358) and patients with lung cancer (median 226 pg/mL, IQR 33–1043) or patients with early-stage lung cancer (median 753 pg/mL, IQR 172–1957). However, there was a significant difference when comparing control subjects and patients with late-stage lung cancer (median 161 pg/mL, IQR 33–643) ($p < 0.01$).

Table 1. Descriptive characteristics of participants.

Variable	Control subjects	Patients with lung cancer
Sex (women/men)	83/88	39/40
Age, years	64 (17)	68 (16)
Tobacco pack years	15 [0–40]	35 [20–48]
Ever-smoker	120 (70%)[#]	72 (91%)
Never-smoker	49 (29%)[#]	7 (9%)
Stage I–II vs. III–IV	NA	29 (37%) vs. 50 (63%)

Presented as frequencies (%), mean (standard deviation) or median [interquartile range]. [#]Smoking status was not available (NA) for two subjects.

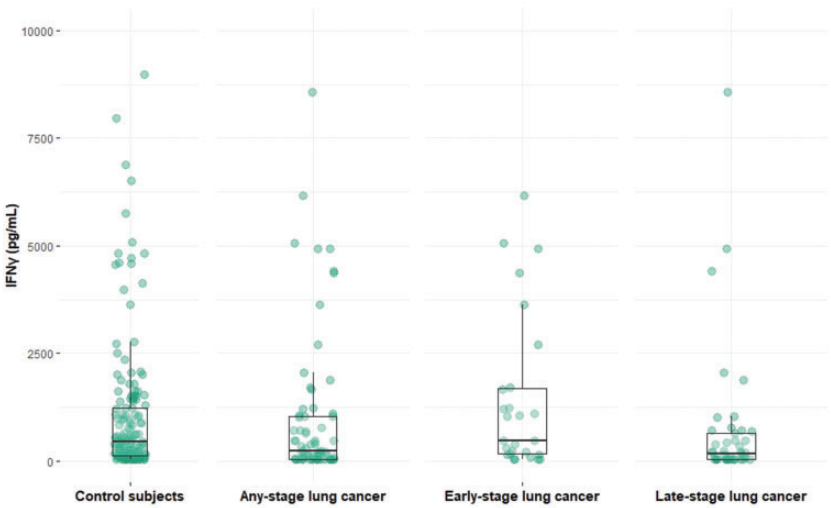

Figure 1. Boxplots displaying interferon gamma (IFNγ) release in different patient groups. IFNγ plasma levels were measured by an enzyme-linked immunosorbent assay. Outliers above 10,000 pg/mL ($n = 5$) are not depicted but included in the statistical analyses.

Similarly, the levels of NKA measured in those with early-stage lung cancer and late-stage lung cancer were statistically different ($p < 0.01$).

The performance of IFNγ release as a predictor of lung cancer diagnosis was evaluated using ROC curve analysis (Figure 2a). The area under the curve (AUC) was 0.57 for the diagnosis of lung cancer ($p = 0.08$), yielding an optimal cut-off value of 240 pg/mL, a sensitivity of 52% and a specificity of 64%. When using the same analysis to assess the detection of late-stage lung cancer, the AUC was 0.66, with an optimal cut-off value of 240 pg/mL, sensitivity of 62% and specificity of 64% ($p < 0.01$) (Figure 2b). Similar results were obtained for previous and present smokers, with an AUC of 0.57 for the diagnosis of lung cancer and 0.65 for the detection of late-stage lung cancer.

The odds of being diagnosed with lung cancer or late-stage lung cancer for participants in the cohort were evaluated (Figure 3). Participants in this high-risk cohort had an odds ratio of 1.8 [95% confidence interval (CI): 1.0–3.4; $p = 0.06$] and 1.8 [95% CI: 1.1–3.2; $p < 0.05$] for being diagnosed with any stage of lung cancer at the cut-off values of 120 pg/mL (the lower reference limit) and 240 pg/mL (optimal cut-off value), respectively. The odds ratio for having late-stage lung cancer was 2.9 [95% CI: 1.5–5.8; $p < 0.01$] and 2.7 [95% CI: 1.4–5.2; $p < 0.01$] at the cut-off values of 120 pg/mL and 240 pg/mL, respectively.

Influence of clinical factors on NKA

To assess whether age, sex and smoking status influenced the release of IFNγ, a multiple linear regression model was constructed containing all participants. As shown in Table 2, none of these clinical factors were found to be associated with IFNγ release in this high-risk cohort.

Discussion

Numerous studies have shown that NKA is downregulated by tumors.[11,12,15] We aimed to investigate whether this deleterious impact on the immune system could be used for diagnostic purposes. Specifically, we studied if the level of IFNγ produced by stimulated NK cells predicted lung cancer in a high-risk cohort.

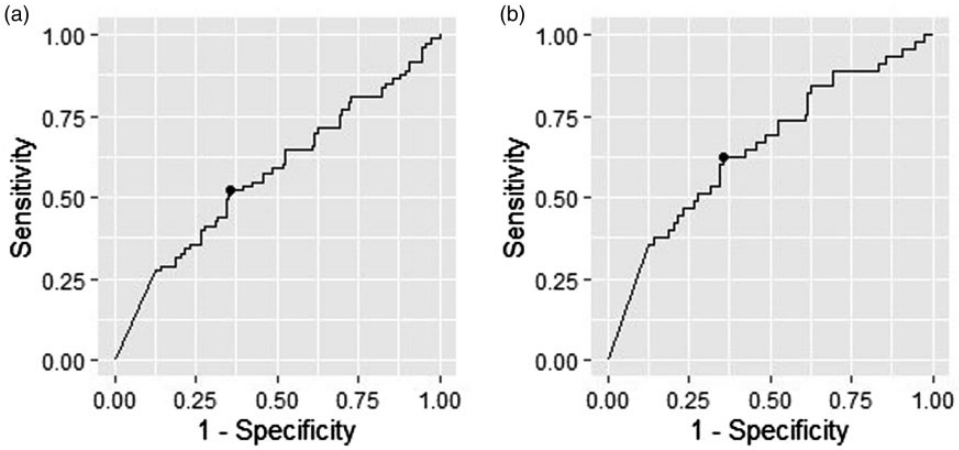

Figure 2. Receiver operating characteristic curve of natural killer cell activity for the diagnosis of (a) lung cancer [area under the curve (AUC) = 0.57] and (b) late-stage lung cancer (AUC = 0.66).

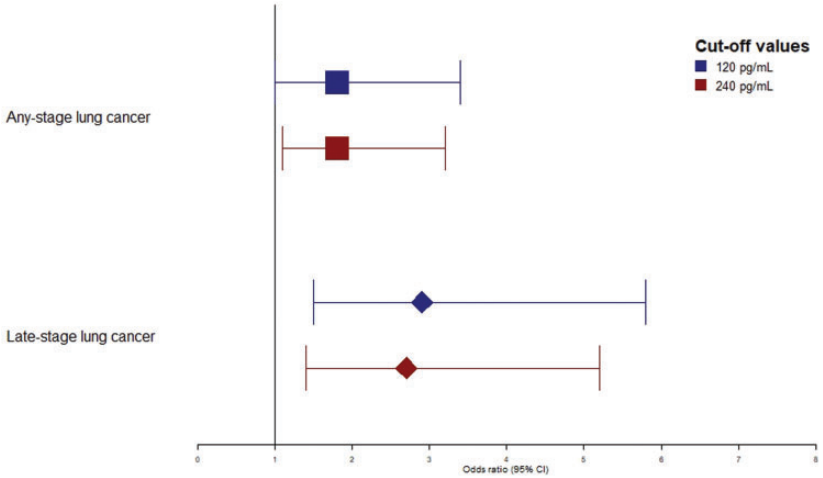

Figure 3. Odds ratio and 95% confidence interval (CI) of lung cancer or late-stage lung cancer diagnosis at different cut-off values of interferon γ release (pg/mL).

Table 2. Influence of clinical factors on NK cell activity.

Variable	β-coefficient	p-value
Men	−116.2 [−817.5–585.1]	0.74
Ever-smoker (vs. never-smoker)	235.9 [−1093.8–622.0]	0.59
Age	11.9 [−19.9–43.6]	0.46

Data are presented as the β-coefficient with the 95% confidence interval.
NK, natural killer.

Notably, although there was no significant difference in NKA between control subjects and patients with any-stage lung cancer or early-stage lung cancer, the difference was highly significant between control subjects and patients with late-stage lung cancer in this study. This is similar to previous reports.[15] Correspondingly, the AUC of the ROC curve and sensitivity/specificity were improved when performing analyses for the detection of late-stage lung cancer. This result is consistent with previous studies that found a negative correlation between NKA and tumor stage,[22,23] and it may explain why NKA appears to be most valuable in the diagnosis of late-stage lung cancer in our high-risk cohort.

The estimated cut-off value in this high-risk cohort was 240 pg/mL. This is similar to the recommended cut-off value of 250 pg/mL for NKA predefined by the manufacturer (NKMAX, South Korea). Conceivably, the optimal cut-off value may vary depending on the sample and type of cancer investigated. Nonetheless, the cut-off value obtained in this lung cancer study appears to be similar to those stated in other publications, including 391 pg/mL in a separate lung cancer study,[15] 218 pg/mL in prostate cancer,[24] 181 pg/mL in colon cancer[11] and 438 pg/mL in gastric cancer.[12] These reports support our findings and indicate that an identical cut-off value may sufficiently separate malignancy from normal in the diagnostic setting of solid tumors.

Sensitivity and specificity at the optimal cut-off value in this study were found to be

52% and 64%, respectively. In other organ-specific cancer types, the assay has yielded diverse diagnostic properties, with sensitivity/specificity ranging between 87%/61% in colon cancer,[11] 67%/92% in gastric cancer[12] and 34%/88% in prostate cancer.[24] A previous study by Choi et al[15] assessing the diagnostic properties in non-small cell lung cancer found a sensitivity of 50.8% and specificity of 89.1% when using patients with benign lung disease as a reference. Hence, compared with our results, the sensitivities of the assays were similar, but the specificity was markedly higher in the study by Choi et al. Differences in the design of the two studies may partly explain this discrepancy. In the previous publication, the benign lung disease group consisted of 40 selected patients diagnosed with chronic obstructive pulmonary disease (35%), asthma (32.5%), tuberculosis (10%), idiopathic pulmonary fibrosis (10%), bronchiectasis (7.5%) and pneumonia (5%). In contrast, in the current study, the control group included participants in which lung cancer had been ruled out after diagnostic investigation. Because these reference groups were not comparable in terms of the diagnoses or number of enrolled patients, a direct comparison between the two is difficult. Furthermore, because the controls in this study were part of a high-risk group under suspicion of cancer but turned out not to have cancer, they may be considered more resistant to cancer than the general population. The rationales of NKA measurements in both studies were to distinguish patients with lung cancer from healthy at-risk participants with similar risk profiles; therefore, we believe the current design is superior in this context.

The current study found no influence of age, sex or smoking status on NKA. However, previous work has provided conflicting results regarding the impact of these clinical factors. Measuring NKA as either NK cell cytotoxic capacity or NK cell ability to secrete IFNγ, two studies in healthy participants found that NKA was significantly lower in smokers by direct comparison.[25,26] Moreover, research investigating the impact of age on NKA revealed impaired IFNγ secretion in the elderly, while the cytotoxic capacity was comparable across age groups.[27] Additionally, a study using the same NK Vue® assay as our analysis found no association with age, sex or smoking in a linear regression model consisting of a population of control subjects and patients with cancer, similar to the current study.[15] This may suggest that in the context of cancer, the impact of age and smoking status becomes insignificant, whereas in healthy cohorts, a large variation in age distribution and smoking status may give rise to differences in NKA. However, this needs to be thoroughly investigated in a study that takes health, age and smoking status into consideration and uses a standardized assay to enable the comparison of results. This is crucial as comparisons between the studies mentioned above are difficult because of differences in techniques applied for NKA measurements. Other clinical factors that may influence NKA include autoimmune diseases and likely the use of anti-inflammatory medication.[28] Unfortunately, information on these factors for each patient was not accessible. Hence, we were unable to determine if they impacted the outcome of tests.

Based on the current study, NKA measurements appear to show potential as a diagnostic marker in late-stage lung cancer. It is likely that the performance will improve if NKA is combined with other relevant biomarkers, such as autoantibodies, carcinoembryonic antigen (CEA), C-terminus of cytokeratin 19, cancer antigen-125 and pro-surfactant protein B measured in serum.[29,30] In gastric cancer, a combination of NKA, CEA and/or cancer antigen 19-9 improved sensitivity

compared with each biomarker alone.[12] In contrast, NKA alone does not appear suitable for screening purposes as it was unable to distinguish patients with stage I and II lung cancer from control subjects. Furthermore, in this study, NKA was significantly lower in patients with disseminated cancer than in control patients, indicating its potential use as a marker of treatment effects in disseminated cancer,[19] a predictor of immunotherapy response in disseminated lung cancer[31] or for relapse surveillance,[32] as suggested previously. Thus, further investigation of the use of NKA as a supportive diagnostic marker is needed.

Certain limitations apply to this study. The number of participants causes a risk of type II error, and because of the setup of the study, control subjects and lung cancer cases were not matched in smoking habits or age. However, this setup is also a strength of the study, as it matches clinical settings. Moreover, some clinical factors not considered in the analyses, such as autoimmune diseases and anti-inflammatory medicine, may impact the data. The influence of these factors should be thoroughly investigated prior to the use of NKA as a diagnostic tool in the clinic.

The present study aimed to evaluate the use of NKA for the detection of lung cancer in a high-risk cohort. In conclusion, we found significantly lower NKA in patients with late-stage lung cancer compared with that in both participants in which lung cancer was ruled out and patients with early-stage lung cancer. The optimal cut-off value for IFNγ release was in the same range as that reported in previous studies. The diagnostic yield of the assay was best in the detection of late-stage lung cancer, which also makes it less suitable for screening purposes. Future research could focus on combining NKA with other biomarkers relevant for lung cancer diagnosis and examining the potential role of NKA as a marker of treatment effects in disseminated diseases or as a surveillance tool for relapse. This would require a large-scale study of late-stage lung cancer.

Acknowledgements

The authors would like to thank the Danish National Centre for Lung Cancer Research for their excellent support, Kristina Kock Hansen for identifying potential participants and managing the database, Anne-Mette Gintberg, Marianne Mikkelsen, Rikke Maria Iversen and Marianne Kammer for enrolling the participants, Karin Larsen for managing funding and ethics board approval, Nilosa Ushanthan, Sara Egsgaard and Camilla Davidsen for performing the laboratory analyses and Sandra Esperanza Rubio-Rask for entering data into the database.

Author contributions

(I) Conception and design: MB, LN, OH, TH, SW, RA
(II) Administrative support: UMW, OH
(III) Provision of study materials or patients: LN, OH, RA, AJ
(IV) Collection and assembly of data: SW, LN, RA, TH, AJ
(V) Data analysis and interpretation: MB, OH, UMW, LN
(VI) Manuscript writing: All authors
(VII) Final approval of manuscript: All authors

Declaration of conflicting interest

All authors have completed the ICMJE uniform disclosure form. AJ received research funding from NKMAX Co. Ltd.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grants from the Region of Southern Denmark (J.nr. 20/14276, Efond 481), Gangstedfonden (grant number A35818) and Lillebaelt Hospital Research Foundation. AJ received research funding from NKMAX Co., Ltd. The sponsors had no role in the study design, collection, analysis and

interpretation of data, writing of the report and decision to submit the article for publication.

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