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Identifying Serum Metabolite Biomarkers Of Thyroid Cancer

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Identifying serum metabolite biomarkers of thyroid cancer

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Abstract

**Background:** In recent years, the incidence rate of thyroid cancer is increasing rapidly, including a significant increase in young aged group people. Environmental chemical exposure is a significant driver. With untargeted metabolomics approach, this study will perform an untargeted metabolomics approach to identify serum metabolites biomarkers associated with thyroid cancer and tumor size.

**Methods:** Serum samples of 100 thyroid cancer patients (50 cases and 50 controls) diagnosed between 2010 and 2011, were extracted for metabolites and analyzed by reversed-phase liquid chromatography-mass spectrometry (LC-MS) based untargeted metabolomics. All the analysis were conducted in both positive and negative Electrospray ionization (ESI) modes. Multivariate and univariate data analysis tools are applied to identify metabolic differences among different sample groups and metabolites associated with the thyroid cancer cases.

**Results:** No significant metabolic difference through multivariate analysis were observed among different sample groups (small case, large cases and controls). One metabolite methylcytosine [M-H2O-H] was identified through univariate analysis in the ESI negative mode when comparing cases (small cases and large cases) and controls, which can be caused by environmental exposure.

**Conclusions:** There were no significant metabolic differences among different sample groups. One metabolite methylcytosine [M-H2O-H] of thyroid cancer was identified. Future research with a larger sample size and measurement of environmental chemicals in cancer patients are needed to uncover the association between environmental chemical exposure and thyroid cancer.

**Keywords:** Thyroid cancer, Environmental Chemicals, Endocrine disrupting, Untargeted metabolomics, Biomarkers
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1. Introduction

In recent years, the incidence rate of thyroid cancer (mainly for papillary thyroid carcinoma) is increasing rapidly, and it is also the fastest increasing cancer among all the cancers in the US [1]. According to the US Surveillance, Epidemiology and End Results, the incidence increase rate of thyroid cancer is 3.6% per year on average from 1974 to 2013, and it has increased more than three times from 4.56 per 100,000 person-years (1974-1977) to 14.42 per 100,000 person-years (2010-2013) [2]. Thyroid cancer is also estimated to be the fourth cancer type for diagnosis in the US by 2030, and the societal cost is estimated to exceed $3.5 million by 2030 [3,4]. Moreover, there is a significant increase of thyroid cancer incidence among young aged people, who don’t usually have imaging exams [3, 5]. It is very important to find out the underlying causes about the rapid increase of thyroid cancer incidence for thyroid cancer prevention strategies.

Although improved diagnostic imaging methods are partly responsible for the increase of the thyroid cancer, it can only explain around 50% of the increase in recent decades [6]. The etiology and risk factors for thyroid cancer haven’t been well-understood yet and still remains largely unknown. Currently, one well-established risk factor driving the increase of thyroid cancer is ionizing radiation especially during childhood [7]. There are also many other possible factors, such as family history of thyroid cancer, dietary habits, lifestyle change, excess bodyweight and height, sex, iodine intake and other risk factors [4,7,8]. In particular, many studies have indicated that environmental chemical exposure is a significant potential driver for the increase of thyroid cancer, given their wide presence and high dispersion in the environment [3, 9, 10, 11, 12].
Most of the chemicals in the environment and consumer products are endocrine disrupting chemicals (EDCs), including organophosphate flame retardants (PFRs), polybrominated diphenyl ethers (PBDE), polybrominated biphenyls (PBB), polychlorinated biphenyls (PCBs), perfluorinated compounds (PFCs), bisphenol A (BPA), phthalates, organophosphate pesticides and so on [13]. In human studies, several of these EDCs have shown their thyroid disrupting properties, and the disruptive activities include disturbance of thyroid hormones synthesis, interaction with the thyroid hormone receptor and secretion and metabolism [8]. In addition, thyroid is very sensitive and susceptible to EDCs, and tend to develop thyroid cancer [14]. However, there is still a lack of data and information about the carcinogenic effects of EDCs, and there is also an urgent need to understand the potential mechanisms of EDCs impacting human health.

The novel hypothesis is that the increasing incidence of thyroid cancer is due to the influence of environmental exposure causing procarcinogenic biology in the individuals. However, in this study, I will only use an untargeted metabolomics approach (which can identify environmental and endogenous metabolites) to identify metabolites associated with thyroid cancer and tumor size, in order to identify any differences in metabolism among different sample groups (small case group, large case group and control group) and also identify non-invasive serum biomarkers of thyroid cancer. Moreover, as for the next step, the environmental chemicals in thyroid cancer patients will be measured, and the environmental chemical exposure will be linked with some of the metabolites identified in this study. Finally, the correlations between exposure metabolites and endogenous metabolites will be conducted to assess if the environmental metabolites alter
the biology of the patients. The data analysis results will be interpreted for prevention/diagnosis strategies of thyroid cancer as the exposome approach can help identify the early signs and mechanisms of thyroid cancer.

The aim of this study is to identify the serum metabolite biomarkers of thyroid cancer with untargeted metabolomics data, which can help uncover the association between environmental chemical exposure and thyroid cancer risks [15].

2. Review of Relevant Studies
Currently, there are many studies on the association between environmental chemical exposure (especially exposure to ECDs) and the risk of thyroid cancer. Most of the current studies are animal studies and epidemiological studies, including case-control studies, cohort studies, and cross-sectional studies. The most commonly studied chemicals include polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), perchlorate, bisphenol-A, phthalates, pesticides, perfluoroalkyl substances (PFASs) and heavy metals (like cadmium, chromium, lead, mercury). Their mechanisms of thyroid disrupting activities include alteration of blood stream transport, alteration of liver metabolism, alteration of biosynthesis, and all steps of thyroid hormone physiology [16]. The common methods for assessing environmental chemical exposure in these studies include chemical concentrations in serum/urine samples, metabolites and questionnaires [4, 16, 17]. However, to my best knowledge, there are no study using metabolomics approach to uncover the association between environmental chemicals and thyroid cancer. In terms of the results, there are positive associations between thyroid cancer risks and environmental chemicals, like heavy metals (Cu, Pb and Cd), certain
flame retardants, phthalates, bisphenols, and certain pesticides [13, 16]. However, as for other chemicals like PCBs and pesticides, the findings are not consistent and controversial [16].

However, there are several limitations of animal studies and epidemiological studies for finding the link between environmental chemical exposure and thyroid cancer risk. These studies only covered a small proportion of environmental chemicals, and they are often only targeted on one single chemical exposure every time [18]. Also, they didn’t assess the long-term health impacts caused by the environmental chemicals (with exposure changes overtime and the development process of thyroid cancer in human body) [13, 16]. However, the population has usually been exposed to a mixture of different chemicals in the environment and over a long time period, which might not be well reflected by current approaches (animal studies and epidemiological studies). There is still a lack of long-term study of most of the chemicals, as most of them only have been studied sporadically [16].

Therefore, the approach of nontargeted metabolomics is very important to be applied to identify serum metabolite biomarkers in thyroid cancer patients, which can help uncover the link between environmental chemicals and thyroid cancer as it includes the biocontamination information over a long time.
3. Research Design

Serum samples were collected from 100 individuals (50 cases and 50 controls) in the Connecticut thyroid cancer population-based case-control study. Eligible cases were histologically confirmed (papillary, follicular, medullary, and anaplastic) incident thyroid cancer cases, which are diagnosed between 2010 and 2011 and identified through the Yale Cancer Center's Rapid Case Ascertainment Shared Resource, part of the CT Tumor Registry. Among all the 50 cases, there are 24 small cases (tumors < 1cm), including 2 men and 22 women; and 26 large cases, including 8 men and 18 women. Among all the 50 controls, there are 10 men and 40 women. With a metabolome wide association study, the way how environmental exposures correlate with metabolites will be determined, therefore identifying new causal pathways linking exposures to thyroid cancer.

Metabolomics data that will be analyzed in this article was previously acquired by the Dr. Johnson’s Lab in 2019. Briefly, the serum samples (50uL each) were extracted for metabolites and analyzed by reversed-phase liquid chromatography-mass spectrometry (LC-MS) based untargeted metabolomics. Quality control (QC) examples are prepared and analyzed with the same procedure. The QC samples contains a small amount of each of the samples that were analyzed, and they were analyzed multiple times repeatedly in the LC-MS analysis to make sure the system is working well and the metabolic data is of high quality. All the analysis above were conducted in both positive and negative Electrospray ionization (ESI) modes. Data was processed using xmcs (22) and CAMERA (49) packages in R. Data was normalized using support vector regression (50) and available in .csv format for statistical analysis.
Statistical Analysis

Multivariate and univariate data analysis tools are applied to identify metabolites associated with the thyroid cancer cases.

Principal component analysis (PCA) is used to reveal the differences of metabolic profiles of serum samples among small cases, large cases and controls. The reliability of the model is determined by $R^2$ value (explanatory rate) and $Q^2$ value (forecast rate). $R^2$ value represents the percentage of explained variance within the model, and $Q^2$ value represents the percentage of predicted variation within the model. And the model is more reliable with higher $R^2$ and $Q^2$ values (0.6-1.0, and the difference between $R^2$ and $Q^2$ is no more than 0.2).

Wilcoxon analysis is conducted to identify the significant metabolites, with adjusted $p$ value < 0.05 at the significance level. Volcano plots were conducted to show the visualization, and the fold-change and $p$ values were log transformed.

All analysis is performed with R studio (Version 1.4.1106). Metabolites are identified with an in-house database and comparison of mass spectral information to additional databases such as METLIN (36).

4. Results
4.1 Serum untargeted metabolomics analysis in positive ESI mode
4.1.1 Multivariate data analysis
In the positive ESI mode, Figure 1 shows that the QC samples in the PCA model are well clustered, which indicate good reproducibility in the LC-MS analysis. Even though the QC samples should be in an identical position within the plot in theory, there hasn’t been any instrumental drift, and it can still indicate the stability of the mass spectrometry run and the good quality of the data.

The model statistic $R^2$ values are 0.085 and 0.043 for PC1 and PC2, respectively. And $Q^2$ values are 0.063 and 0.084 for PC1 and PC2 respectively. $R^2$ and $Q^2$ values are quite low, which indicates that the model doesn’t show metabolic variation between different sample groups.

![PCA on Thyroid Cancer Positive Mode](image)

*Figure 1 PCA scores plot with quality control (ESI positive mode)*

In Figure 2, the blue circle, orange triangle and gray cross represent the large case group, the small case group and the control group, respectively. There is a relative dispersion of three groups of samples, and the samples are clustered within their groupings, indicating differences in serum metabolome in different groups. Each blue circle represents one of
the large tumors analyzed, and all the blue circles are quite clustered tightly, so their metabolomic profiles are more similar than the controls or smaller tumor case group. The smaller cases (orange triangles) are a little more dispersed within their grouping, but still residing in the same space. However, the controls (gray cross) are relatively more dispersed on the plot but still grouped together, indicating that the metabolic profiles are more varied among health controls.

The explained variances on PC1 and PC2 are quite low. The models don’t explain much variation between the controls and cases, as the total data variance explained by PCA model is only 12% (PC1 = 8% explained variance, PC2 = 4% explained variance). The model statistic $R^2$ values are 0.085 and 0.043 for PC1 and PC2, respectively. $Q^2$ values are 0.061 and 0.0068 for PC1 and PC2 respectively.

It’s interesting to observe that there is visual separation in metabolomes between controls, and cases by tumor size; but the percentage of variance explained by the model is low. This may be due to the small sample size (n = 100) in this study, and further researches are needed to investigate more about the metabolic variation among different sample groups (small case, large case and control) with a larger sample size.
4.1.2 Univariate data analysis

Wilcoxon analysis is applied to identify the significant metabolites, and volcano plot is used to visualize the statistical results and identify the significant metabolites between the case group (small and large cases) and control group. Figure 3 shows that there is no significant metabolite between the case group and control group after multiple comparisons. Moreover, as the control group and large case group are more separated in the plot, they were also compared (Figure 4). However, there is still no significant metabolite observed.
4.2 Serum Metabolomics profile analysis in negative ESI mode

4.2.1 Multivariate data analysis

In the negative ion mode, Figure 5 shows that the QC samples in the PCA model are well clustered, which indicate good reproducibility in the LC-MS analysis and good data quality, although the QC samples are not in an identical position within the plot.
The model statistic $R^2$ values are 0.075 and 0.065 for PC1 and PC2, respectively. $Q^2$ values are 0.026 and 0.069 for PC1 and PC2 respectively.

In Figure 6, the blue circle, orange triangle and gray represent the case group with large tumors, the case group with small tumors and the control group, respectively. In terms of visual inspection, there is not an obvious dispersion of three groups of samples, indicating that there are not obvious differences in serum metabolome in different groups. There is also almost no separation between small cases and large cases, indicating that there is no metabolic variation between small cases and large cases. However, the separation between the case group (small cases and large cases) and control group is observed, indicating that there might be metabolic variation between case group and control group.

The explained variances on PC1 and PC2 are also quite low in the negative ESI mode. The models don’t explain much variation between the controls and cases, as the total data...
variance explained by PCA model is 15% (PC1 = 8% explained variance, PC2 = 7% explained variance). The model statistic R² values are 0.076 and 0.066 for PC1 and PC2, respectively. Q² values are 0.030 and 0.074 for PC1 and PC2 respectively.

4.2.2 Univariate data analysis

The volcano plot (Figure 7) shows that there are two significant metabolites between the case group (small cases and large cases) and control group after multiple comparisons. In our analysis, the univariate analysis generated a table with FDR-corrected p-values, the identifiers in the table are m/z = 106.0417, wilcox.p value = 5.29E-14, wilcox. adjusted p values (fdr) = 6.57E-10, fold change difference between cases and controls = 0.299684; and m/z = 408.283, wilcox.p value = 1.16E-13, wilcox. adjusted p values (fdr) = 7.21E-10, fold change difference between cases and controls = 2.706164.

The putative identification for metabolite 106.0417 is methylcytosine [M-H2O-H]. The mass error 6.19 ppm, and it’s upregulated 2.7-fold in the thyroid cancer cases. Moreover,
this metabolite is a biomarker for DNA damage, which can be caused by environmental exposures.

There are a few putative identifications for metabolite 408.283. However, the identifications might only be putative, because the mass error is larger (> 13 ppm). For example, some identifications to METLIN with a mass error below 20 ppm include 3,5-Didecanoylpyridine (mass error 13.39 ppm); 3-Hydroxy-10'-apo-b,y-carothenal (mass error 19.27 ppm); and 20, 22-Dihydrodigoxigenin (mass error 18.1ppm). However, the identification of metabolite 408.283 needs further investigation.

![Volcano plot of metabolic differences between cases and controls (ESI negative mode)](image)

Figure 7 Volcano plot of metabolic differences between cases and controls (ESI negative mode)

5. Discussion

In this study, the serum samples were analyzed in both positive and negative ESI modes to examine different metabolites within the widest range of metabolites possible. And two
significant metabolites were found in the negative mode when all cases (small cases and large cases) and controls were compared.

In the positive ESI mode, there is a dispersion of different sample groups (small case, large case and control) by visual examination of the PCA model, indicating trends of metabolic differences among different groups. However, the total variance explained by PCA (12%) is low. Moreover, none of the metabolites were statistically significant. The reason might be that this study is under power with the small sample size (n =100), and future research are needed to further examine the metabolic differences among different sample groups (small cases, large cases and control) with a larger sample size, and also identify significant metabolites.

In the negative ESI mode, there is a dispersion of the cases (small tumor and large tumor) and controls by visual examinations of the PCA model; however, there is almost no dispersion between small case group and large case group. This indicates that there might be metabolic differences between the cases and controls. However, the total variance explained by PCA (15%) is also relatively low. Similar to the positive mode, the reason might be that this study is under power with a small sample size (n =100). Further researches are needed to examine the metabolic differences between controls and all the cases (small tumor and large tumor) with a larger sample size.

And in the negative mode, two metabolites were identified to have the association with thyroid cancer (metabolite 106.0417 and metabolite 408.283) when cases (small tumor and
large tumor) and controls were compared. The putative identification for metabolite 106.0417 is methylcytosine [M-H2O-H], which is a biomarker for DNA damage and can be caused by environmental exposures. Further, we still need to confirm the identification in Dr. Johnson’s Lab. However, the identification of metabolite 408.283 may only be putative (mass error >13 ppm) and still needs further investigation.

Moreover, methylcytosine [M-H2O-H] has also been proved to be a marker to monitor DNA methylation [19]. And DNA methylation has been proved as epigenetic changes for many existing environmental chemicals, including arsenic, nickel, chromium, mercury, lead, BPA and pesticides [20]. Also, methylcytosine [M-H2O-H] has also been previously reported as a biomarker of DNA damage, and it can be caused by environmental chemical exposure.

There are several studies on the metabolomics of thyroid cancer appearing in the past few years, and the three most referred metabolites are choline, lactate and tyrosine, and other identified metabolites include leucine, lysine, phenylalanine, citrate, and so on [21]. These metabolites are different from the two metabolites identified in my study, and this might be due to different methods, study groups, study site, samples (like serum and tissue) and different sample size used in different studies, and future research can be conducted with more similar populations and samples and a larger sample size (higher possibility to identify more significant metabolites).
The Importance of this study

It’s also very interesting to notice the rapid increase of thyroid cancer cases in the past few years especially among the young aged group, as there is not much change happening among the population in the past few decades. It is hard to find out the causes, but it is very important to find out the underlying biology and develop thyroid cancer prevention and diagnosis strategies. Environmental chemical is a potential big risk factor due to its thyroid disrupting activities and huge surge in production and application (household products, agriculture, buildings, food packaging, drinking water, medical product, and so on) in the last few decades [22]. The existence of artificial chemicals is ubiquitous in the environment, and the exposure pathways can be through skin contact, diet, inhalation of household dust, air pollution [13, 16]. The novel hypothesis is that the increasing incidence of thyroid cancer is due to the influence of environmental exposure causing procarcinogenic biology in the individuals. In this study as the first step, the serum metabolites associated with thyroid cancer and tumor size were identified, in order to identify any differences in metabolism among different sample groups (small case group, large case group and control group) and also identify non-invasive serum biomarkers of thyroid cancer.

Moreover, the individuals are usually exposed to different kinds of environmental chemicals at the same time, and there is also a biological development process of thyroid cancer over time. However, most of the existing studies (such as animal studies and epidemiological studies) mainly focus on one specific environmental chemical. Untargeted metabolomics is an innovative approach to measure the environmental chemical exposure
accurately, understand the disease aetiology with individual exposotype, and help uncover the association between environmental chemicals and thyroid cancer, for the benefit of thyroid cancer prevention and diagnostic strategies in the public health practice [20].

6. Conclusions

6.1 Summary of findings
There are potential metabolic differences between the case group (including small cases and large cases) and the control group of thyroid cancer by visual examination of PCA model. Moreover, there are also potential metabolic differences between the small case group and large case group of thyroid cancer by visual examination of PCA model. However, in both positive and negative modes, the total variances explained by PCA are relatively low. This might be explained by the low power of the study (sample size = 100), and future researches are needed to investigate the metabolic differences between cases and controls and also cases by tumor size using a larger sample size.

The study has identified two significant metabolites. The certain one is metabolite methylcytosine [M-H2O-H]. It is related to DNA damage, and it can be caused by environmental chemical exposure. Furthermore, it’s also important and potential to identify more significant metabolites with a larger sample size in future research.

Considering the long-term exposure to mixed environmental chemicals of the population, untargeted metabolomics is an important and innovative approach to measure the environmental chemical exposure accurately, understand the disease aetiology with
individual exposotype, which can help uncover the association between environmental chemicals and thyroid cancer, for the benefit of thyroid cancer prevention and diagnostic strategies in the public health practice

6.2 Limitations

Although there is dispersion of different sample groups observed by visual inspection in the PCA model, the $R^2$ values and $Q^2$ values are very low in both positive and negative ESI modes. This might be explained by the power of the study with a sample size of 100. To further investigate the metabolic difference, a larger sample size of serum samples of thyroid is needed for future research.

The numbers of men and women in each sample group are not equal. In the small case group, the ratio of men to women is 1:11; in the large case group, the ratio of men to women is 4:9; and in the control group, the ratio of men to women is 1:4. Although there is a higher thyroid cancer incidence in women than men and all the samples are at the same age, the separation of different sample groups in the PCA model might be driven by gender difference instead of metabolic information difference. Future research is needed to make sure the numbers of men and women are equal in each sample group.

6.3 Recommendations for further research

I’ve found potential metabolic differences between different sample groups by visual inspection in the PCA model (even though total data variance explained by PCA is low), and also identified two significant metabolites. However, the sample size (n=100) in the study might have limited the significant metabolites of thyroid cancer. It’s very important to increase sample size in the future and hopefully identify more significant metabolites.
The biomarker methylcytosine [M-H2O-H] found in the study indicates that the pathway that environmental chemicals might cause thyroid cancer is through DNA damage. As it is putative identification, we still need to confirm it in Dr. Johnson’s lab. It’s important for future research to further prove the link of DNA damage between different environmental chemicals and thyroid cancer.

In this study, I only conducted the first step of identifying serum metabolites of thyroid cancer patients. Future researched are needed to measure environmental chemicals in patients who have thyroid cancer. The rationale is that the chemicals might effect tumor progression or prognosis (they could effect cellular processes such as immune responses, or they could have other signaling/metabolic effects that could propagate tumor growth).

The environmental chemical exposure will be linked with some of the metabolites identified in this study. Moreover, the correlations between exposure metabolites and endogenous metabolites will be conducted to assess if the environmental metabolites alter the biology of the patients.
References:


