A novel autoantibody against moesin in the serum of patients with MPO-ANCA-associated vasculitis

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ABSTRACT

Background. Antineutrophil cytoplasmic autoantibody (ANCA) directed against myeloperoxidase (MPO), a diagnostic criterion in MPO-ANCA-associated vasculitis (MPO-AAV), does not always correlate with disease activity. Here, we detected autoantibodies against moesin, which was located on the surface of stimulated endothelial cells, in the serum of patients.

Methods. The anti-moesin autoantibody titer was evaluated by ELISA. Seventeen kinds of cytokines/chemokines were measured by a Bio-Plex system.

Results. Serum creatinine in the anti-moesin autoantibody-positive group was higher than that in the negative group. Additionally, interferon (IFN)-γ, macrophage chemotactic peptide-1 (MCP-1), interleukin (IL)-2, IL-7, IL-12p70, IL-13, granulocyte/macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor were significantly higher in the positive group. Furthermore, IL-7 and IL-12p70 levels correlated with the anti-moesin autoantibody titer. Based on these findings and the binding of anti-moesin IgG to neutrophils and monocytes, we detected the secretion of cytokines/chemokines such as IFN-γ, MCP-1 and GM-CSF from these cells.

Conclusions. The anti-moesin autoantibody existed in the serum of patients with MPO-AAV and was associated with the production of inflammatory cytokines/chemokines targeting neutrophils with a cytoplasmic profile, which suggests that the anti-moesin autoantibody has the possibility to be a novel autoantibody developing vasculitis via neutrophil and endothelial cell activation.

Keywords: autoantibodies, glomerulonephritis, moesin, myeloperoxidase, neutrophil

INTRODUCTION

Rapidly progressive glomerulonephritis (RPGN) has been classified as microscopic polyangiitis (MRA) showing myeloperoxidase (MPO)-anti-neutrophil cytoplasmic autoantibodies (ANCA) in the serum of patients. ANCA-associated vasculitis (AAV) is classified by the type of autoantibodies present, one of which is MPO-ANCA, which recognizes MPO in neutrophils [1–3]. Many studies have implied that MPO-ANCA reacts with neutrophils to induce neutrophil infiltration and the secretion of inflammatory cytokines and chemokines [4–6]. When neutrophils are primed with tumor necrosis factor-alpha (TNF-α), MPO is expressed on the surface of neutrophils to react with MPO-ANCA [7, 8]. On the other hand, proteinase-3 (PR3)-ANCA, which reacts within...
neutrophils, was shown to mainly exist in Europeans and Americans according to an analysis by a Japan–UK group [9]. These two types of AAV show differences in pathological hallmark illnesses [10]. Nevertheless, the detailed initiating mechanisms of these diseases are still unknown.

On the other hand, cytokines and chemokines were found to be elevated in the serum of patients with MPO-ANCA-associated RPGN [11, 12]. The up-regulation of cytokines and chemokines is an important mechanism in the progression of this disease because higher levels of cytokines and chemokines exist systemically or in tissues [13]. TNF-α in particular plays a crucial role in the initiation of inflammation in AAV [14]; the anti-TNF-α antibody combats AAV [15]. Similarly, leukocyte chemotactic factors induce neutrophil migration, resulting in infiltration into renal glomeruli [16]; moreover, monocyte chemotactic protein-1 (MCP-1) has been associated with the Th immune response cascade, resulting in more severe glomerulonephritis [17, 18]. However, the trigger factors of cytokine/chemokine secretion in MPO-AAV are unclear.

A high dose of the anti-MPO antibody was shown to induce glomerular damage in mice, leading neutrophils to up-regulate adhesion and infiltration in glomeruli [19, 20]. Furthermore, our previous study showed that the SCG/Kj mouse was suitable as a model for MPO-AAV [21] because it is well-known to have high levels of MPO-ANCA with small vessel vasculitis and crescentic glomerulonephritis. In addition, anti-moesin autoantibodies were shown to be produced in the serum of active phase of SCG/Kj mice [22]. These studies suggested that anti-MPO antibodies are the main factor in the development of MPO-AAV, whereas anti-moesin autoantibodies are concerned with the exacerbation of vasculitis. However, MPO-ANCA has not necessarily been correlated with the exacerbation and/or relapse of the disease [23]. The reasons why this circutious mechanism induces vasculitis by the MPO antibody and the role of anti-moesin autoantibodies are not clearly known.

Recent studies have demonstrated that rabbit-anti-mouse MPO polyclonal antibody (pAb) reacted with mouse glomerular epithelial cells and moesin autoantibodies were measured in human serum by ELISA. Brieﬂy, recombinant full-length moesin was purchased from Abnova Co. (Taipei, Taiwan). Bovine serum albumin (BSA), Tween-20, p-nitrophenyl phosphate, protease inhibitor cocktail, peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated goat anti-human IgG were purchased from Sigma (St. Louis, MO, USA). Alkaline phosphatase-conjugated anti-human IgG antibody was obtained from Jackson ImmunoResearch (Penn, PA, USA). ECL Western Blotting Detection Reagents kit was a product of GE Healthcare UK (Amersham Place, UK).

Patients
Clinical and laboratory data for 60 patients with MPO-AAV ranging in age from 38 to 87 years (average 67.07 ± 12.21, 9 males, 51 females) were examined at Tokyo Women’s Medical University and Jichi University. We also enrolled 31 healthy volunteers ranging in age from 37 to 98 years (average 69.19 ± 15.01, 15 males, 16 females) at Chiba University and Jichi University. This study was reviewed by the Ethical Committee of Chiba University, Tokyo Women’s Medical University and Jichi University Medical School Japan in 2009, and the protocol design was approved.

Isolation of neutrophils and monocytes
Human neutrophils were isolated from peripheral whole blood using one-step polymorphs. Human adhesive monocytes were collected from peripheral blood mononuclear cells (PBMCs). Briefly, human PBMCs were cultured in a 24-well plate (Iwaki, Tokyo) at a density of 3 × 10^5 cells per well with D-MEM with 5% FBS for 4 h. The wells were then washed three times and used as adhesive monocytes.

Determination of anti-moesin autoantibodies
Anti-moesin autoantibody titers were measured in human serum by ELISA. Brieﬂy, recombinant full-length moesin was coated on 96-well plates (Iwaki, Tokyo) at a density of 2 µg/mL with coating buffer (15 µM sodium bicarbonate, 35 µM sodium hydrogen carbonate) overnight at 4°C, and then wells were blocked by PBS with 1% BSA. Diluted human serum (1:50) in PBS with 1% BSA was added to the wells and incubated for 1 h at room temperature, and the wells were then washed three times with PBS with 0.05% Tween-20. The primary antibody was detected by incubation with an alkaline phosphatase-conjugated anti-human IgG antibody (1:1000) for 1 h at room temperature. The bound secondary antibody was subsequently quantified by changes in absorbance at 405 nm after incubation with 1 mg/mL p-nitrophenyl phosphate.

Detection of MPO-ANCA
To measure MPO-ANCA titers in human serum, we contracted out to SRL, Inc.
Western blotting

Neutrophils, PBMCs and recombinant moesin (rm-moesin) were lysed in accordance with our previously report [22]. After centrifugation (20 000 xg, 10 min, 4°C) of the lysate, the supernatant was separated and transferred by subjecting to Nu-PAGE analysis, which was performed with the Novex Nu-PAGE system according to the manufacturer’s instructions [30]. The transferred proteins were reacted with 5 μg/mL mouse anti-moesin pAb, patient serum with MPO-ANCA or serum from healthy individuals. Immunoreactive spots were detected using peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated goat anti-human IgG and ECL Western Blotting Detection Reagents.

Detection of cytokines and chemokines

Human serum and culture supernatants were analyzed for 17 cytokines and chemokines using a multiplex assay according to the manufacturer’s instructions (Bio-Rad, Hercules, CA, USA).

Binding of anti-moesin pAb to the cell surface by flow cytometry

Flow cytometry was performed with a Becton Drive (NJ, USA). Isolated human neutrophils and PBMCs were resuspended in HBSS with 5% FBS. Surface moesin was detected with mouse anti-moesin pAb using Alexa488-conjugated anti-mouse IgG, and normal mouse IgG served as an isotype control. To identify the cell population, anti-CD3, anti-CD11b and anti-CD14 antibodies were reacted with neutrophils or PBMCs. Dead cells in all samples were eliminated by propidium iodide (PI) staining.

Antibody treatment of cells and analysis

Isolated neutrophils or adhesive monocytes were seeded on a 96-well plate at a density of 2 x 10^5 cells per well. To obtain the cell culture supernatant, cells were incubated with control mouse IgG or anti-moesin pAb (10 μg/mL) overnight and the cell culture supernatant was collected for analysis by the multiplex assay with a Bio-Plex system (Bio-Rad, Hercules, CA, USA).

Determination of the recognition profile of the anti-moesin autoantibody in neutrophils by immunofluorescence microscopy

The anti-moesin autoantibody was stained by the FLUORO ANCA test and tested following the manufacturer’s protocol (Medical Biological Lab, Japan). The fluorescence profile was obtained by a Keyence system from Keyence Corporation (Osaka, Japan).

Statistical analysis

All data are expressed as the mean ± SEM. Comparisons between two groups were performed using the Student’s t-test and differences with P < 0.05 were considered significant. Comparisons among more than three groups were performed using the Kruskal–Wallis one-way ANOVA on ranks (P < 0.01) followed by the Mann–Whitney U-test.
especially IL-12p70 and IL-7 were correlated with the anti-moesin titer (Supplementary Figure S2). Similarly, IFN-γ, MCP-1, G-CSF, GM-CSF and IL-2 were higher in the anti-moesin-positive group (Figure 2Ba, b, d, e). The levels of TNF-α were slightly higher (P = 0.053) in the anti-moesin autoantibody-positive group (Figure 2Bc). No significant differences in the other cytokines/chemokines examined were observed between the anti-moesin autoantibody-positive group and anti-moesin autoantibody-negative group (data not shown). In addition, we sorted patient serum by sampling point and also confirmed in laboratory data and cytokines/chemokines. Some cytokines/chemokines in the treatment period and relapsed phase were slightly higher than quiescent phase, and a similar result was carried out in the CRP (Supplementary Figure S3). These results suggest that the anti-moesin autoantibody may be involved in the inflammatory response that advances vasculitis.

Clinical parameters in the four groups of patients grouped by the MPO-ANCA titer and anti-moesin autoantibody

Although the MPO-ANCA titer has been used for diagnosis, it has not always correlated with the disease activities of patients, which suggests that other diagnostic criteria are needed. To explore the usability of the anti-moesin autoantibody as a novel biomarker, we compared the MPO-ANCA titer with the anti-moesin autoantibody titer using lab data and cytokines/chemokines. Patients were separated into four groups...
according to titers higher or lower than 100 EU/mL of the MPO-ANCA titer, and by the anti-moesin autoantibody-positive or anti-moesin autoantibody-negative group: MPO-ANCA low titer/anti-moesin autoantibody-negative group, \( n = 12 \), MPO-ANCA high titer/anti-moesin autoantibody-negative group, \( n = 16 \), MPO-ANCA low titer/anti-moesin autoantibody-positive group, \( n = 28 \) and MPO-ANCA high titer/anti-moesin autoantibody-positive group, \( n = 4 \) (Figure 3A). The MPO-ANCA titer was classified as 100 EU/mL because patients with titers over 100 EU/mL of MPO-ANCA showed high mortality [32–34]. Serum creatinine, blood urea nitrogen (BUN) and proteinuria were higher in the anti-moesin autoantibody-positive/MPO-ANCA low titer group than in the anti-moesin autoantibody-negative/MPO-ANCA high titer group (Figure 3B a, b, d). MIP-1\( \beta \), IFN-\( \gamma \), GM-CSF and IL-13 were higher in the anti-moesin autoantibody-positive/MPO-ANCA low titer group than in the anti-moesin autoantibody-negative/MPO-ANCA high titer group, and TNF-\( \alpha \) were slightly increased in the same manner. In addition, we compared anti-moesin titer with MPO-ANCA in every sampling points; there were no significant differences (Supplementary Figure S4). These results point to the possibility that the anti-moesin autoantibody could be used to assess vasculitis and inflammatory levels from a different aspect to MPO-ANCA.

**Anti-moesin pAb bound on the surface of neutrophils and monocytes**

To assess the affinity of the anti-moesin antibody for leukocytes, we examined the binding of anti-moesin pAb to leukocytes by flow cytometry and immunohistochemistry. Isolated neutrophils strongly bound to anti-moesin pAb, and monocytes weakly bound to anti-moesin pAb (Figure 5A, B). On the other hand, lymphocytes did not bind to anti-moesin pAb (Figure 5C). Additionally, unfixed neutrophils reacted with anti-moesin pAb (Supplementary Figure S5). These results showed that the anti-moesin autoantibody bound to and reacted with the surface of neutrophils and monocytes.

**Human neutrophils and monocytes were stimulated by anti-moesin pAb**

To clarify which cells were producing cytokines/chemokines in the serum of patients, we examined whether anti-moesin pAb induced the secretion of cytokines/chemokines from isolated neutrophils and monocytes by anti-moesin pAb stimulated in vitro. IL-17, as well as IFN-\( \gamma \), MCP-1, IL-8 and GM-CSF, was higher in neutrophils stimulated by anti-moesin pAb than in isotype controls (Figure 6A–E). No significant differences in TNF-\( \alpha \), IL-6 and MIP-1\( \beta \) were observed between neutrophils stimulated by anti-moesin pAb and isotype controls. These cytokines/chemokines were subsets with high levels in the serum with the anti-moesin autoantibody.

Furthermore, we measured the secretion of cytokines/chemokines from adhesive monocytes to see whether other cytokines/chemokines occurred in high levels in the serum of anti-moesin autoantibody-positive patients. When human monocytes were stimulated with anti-moesin pAb, TNF-\( \alpha \), IL-6, IL-8, MCP-1, MIP-1\( \beta \) and GM-CSF were secreted at higher levels than those stimulated by isotype controls (Figure 7A–F). The levels of these cytokines/chemokines were also subsets with high levels in the serum of anti-moesin autoantibody-positive patients. These results suggest that the anti-moesin autoantibody may associate the progression of vasculitis with the production of inflammatory cytokines/chemokines by...
**FIGURE 3:** BUN, serum creatinine and proteinuria levels in the four groups of patients grouped by the MPO-ANCA titer and anti-moesin autoantibody. (A) Patients were divided into four groups by the anti-moesin autoantibody titer: >51.4 units/mL (healthy control titer mean + 2SD) or below and MPO-ANCA titer: >100 units/mL or below. (B) BUN (a), serum creatinine (b), CRP (c) and proteinuria (d). (C) Serum levels of cytokines/chemokines in the four groups of patients: TNF-α (a), MIP-1β (b), IFN-γ (c), GM-CSF (d) and IL-13 (e) in 17 cytokines/chemokines measured. Data were analyzed by statistical methods (Kruskal–Wallis test, Mann–Whitney U-test) and are expressed as the mean ± SEM. *P < 0.05. White bar, MPO-ANCA high titer and anti-moesin autoantibody-positive; hatched bar, MPO-ANCA high titer and anti-moesin autoantibody-negative; grey bar, MPO-ANCA low titer and anti-moesin autoantibody-positive; black bar, MPO-ANCA low titer and anti-moesin autoantibody-negative.

**FIGURE 4:** Immunofluorescence profile of the anti-moesin autoantibody in neutrophils. Human neutrophils were fixed by EtOH (A–C) or by 4% paraformaldehyde and permeabilized by 0.01% Triton X-100. Immunofluorescence analysis stained with the anti-moesin autoantibody-positive serum of a patient (A and D), MPO-ANCA-positive serum (B and E), healthy serum (C and F) (green) and nuclei were stained by Hoechst33342 dye (blue). The scale bar shows 50 μm.
FIGURE 5: Anti-moesin pAb reacted with the surface of neutrophils and monocytes in flow cytometry. Neutrophils, monocytes and T cells were isolated from the peripheral blood of a healthy volunteer. These cells were multicolor analyzed with antibodies against moesin, CD3, CD11b and CD14, respectively, and with PI. Neutrophils were gated by CD11b (A), monocytes by CD14-positive cells (B) and T cells were identified by CD3 (C). All data were negative gated by PI. Anti-moesin pAb (black line) and isotype controls (shadow line) are shown. MFI, mean fluorescence intensity.

FIGURE 6: Induction of the secretion of IFN-γ, IL-17, GM-CSF, IL-8 and MCP-1 from human neutrophils with anti-moesin pAb stimulation. Human neutrophils (n = 5 each condition) were stimulated by anti-moesin pAb, and the supernatant was then collected 24 h after antibody stimulation. IFN-γ (A), IL-17 (B), GM-CSF (C), IL-8 (D) and MCP-1 (E) were among the 17 cytokines/chemokines quantified by Bio-Plex. Data are normalized to the value of isotype control (IgG2a)-stimulation and expressed as the mean + SEM. *P < 0.05 versus the supernatant of mouse IgG-treated neutrophils. White bar, isotype control stimulation; black bar, anti-moesin pAb stimulation.

FIGURE 7: Induction of the secretion of TNF-α, IL-6, GM-CSF, IL-8, MCP-1 and MIP-1β from human adhesive monocytes by stimulation with anti-moesin pAb. Human monocytes (n = 5 each condition) were stimulated by anti-moesin pAb, and the supernatant was collected 24 h after antibody stimulation. IFN-γ (A), IL-17 (B), GM-CSF (C), IL-8 (D) and MCP-1 (E) were among the 17 cytokines/chemokines quantified by Bio-Plex. Data were normalized to the value of isotype control (IgG2a) stimulation and are expressed as the mean + SEM. *P < 0.05 versus the supernatant of mouse IgG-stimulated monocytes. White bar, isotype control stimulation; black bar, anti-moesin pAb stimulation.
binding of the anti-moesin autoantibody to neutrophils and monocytes.

**DISCUSSION**

In the present study, we investigated the association and function of the anti-moesin autoantibody in MPO-AAV. The anti-MPO pAb was shown to activate mGECs via the moesin molecule, as shown in a previous study, because murine MPO shares amino acid homology with human moesin [20, 22]. Moesin molecules on the surface of mGECs are associated with the initiation and/or exacerbation of vasculitis, and the SCG/Kj mouse was shown to have a high level of the anti-moesin autoantibody in the active phase [22]. Unfortunately, human MPO has no homology with human moesin (data not shown). Therefore, we hypothesized that the surface expression of moesin caused the production of an autoantibody against moesin. We measured the anti-moesin autoantibody in the serum of patients with MPO-AAV. As a result, this study showed, for the first time, that the anti-moesin autoantibody existed in the serum of patients with MPO-AAV. The anti-moesin autoantibody has been reported to contribute to the suppression of hematopoiesis in patients with aplastic anemia, which suggests that the anti-moesin autoantibody in human serum mediates immunological responses [35, 36]. Our results show that the anti-moesin autoantibody is concerned with the disease activity of patients with MPO-AAV.

Proinflammatory cytokines including IFN-γ and TNF-α are one of the important factors initiating small vessel vasculitis in patients with AAV [37, 38]. Similarly, chemotaxis of neutrophils and monocytes is strongly associated with the development of ANCA-associated glomerular nephritis [39]. In the present study, we showed that proinflammatory cytokines/chemokines and serum creatinine levels were higher in the anti-moesin autoantibody-positive group than in the negative group. Other reports have proven that serum creatinine is the severity criterion in renal disorders [40]. We showed that several factors associated with vasculitis occurred at high levels in patients with the anti-moesin autoantibody; therefore, the anti-moesin autoantibody is involved in the development and exacerbation of vasculitis. The anti-moesin autoantibody may mediate the secretion of cytokines/chemokines associated with inflammation from neutrophils and monocytes.

The disease activity of MPO-AAV does not necessarily correspond to the MPO-ANCA titer [41]. For this reason, we grouped patients with MPO-AAV into four groups according to their anti-moesin titer and MPO-ANCA titer and then evaluated laboratory data and cytokines/chemokines in the four groups. We found that BUN, CRP, serum creatinine and proteinuria levels were higher in the anti-moesin autoantibody-positive/MPO-ANCA low titer group than in the anti-moesin autoantibody-negative/MPO-ANCA high titer group. Furthermore, some inflammatory cytokines/chemokines were increased in same manner. We did not find for further differences in the anti-moesin titer with MPO-ANCA at every sampling point, because we could not investigate prospective study and follow-up study. We concluded that these results showed a significant correlation between the anti-moesin titer and disease activity in MPO-AAV. Therefore, these results indicate that the condition of MPO-ANCA patients with the anti-moesin autoantibody could deteriorate even if the MPO-ANCA titer is low. The degree of the severity of AAV was also not limited to just the MPO-ANCA titer; the way in which the MPO-ANCA titer was teamed with the anti-moesin titer was also evaluated.

MPO-ANCA and anti-MPO antibodies react with MPO on the surface of neutrophils to induce the production of proinflammatory cytokines/chemokines when cells are primed by TNF-α, fMLF and other cytokines, and this signaling is then associated with vasculitis [42]. On the other hand, the reactivity of the anti-moesin autoantibody against moesin on the surface of neutrophils and monocytes has been unclear. In addition to our findings from a previous study [43], the results of this study show that anti-moesin pAb directly reacted with moesin on the surface of neutrophils and monocytes. Accordingly, the anti-moesin autoantibody may induce signaling that regulates inflammation by reacting with neutrophils. The anti-moesin pAb stimulated human neutrophils to produce the inflammatory cytokines/chemokines IFN-γ, MCP-1, IL-8, IL-17 and GM-CSF. Additionally, inflammatory cytokines were able to activate adjacent neutrophils to secrete a wide variety of cytokines/chemokines. In the present study, activated neutrophils secreted MCP-1 to attract monocytes.

Although MPO-ANCA does not react with monocytes, monocytes play an important role in the induction of inflammation caused by reactions with autoantibodies [44]. However, the present study shows, for the first time, that the anti-moesin antibody directly reacted with moesin on the surface of monocytes. Consequently, TNF-α and IL-6 were up-regulated in monocytes challenged with anti-moesin pAb. In addition, activated monocytes secreted IL-8 and MCP-1, which are chemokines acting in chemotaxis of neutrophils and monocytes, respectively. The results for inflammatory cytokine/chemokine secretion from isolated monocytes showed that anti-moesin pAb reacted with not only neutrophils, but also monocytes, and may regulate the inflammation and development of vasculitis.

In conclusion, we demonstrated here that most MPO-AAV patients had the anti-moesin autoantibody, which is associated with the up-regulation of inflammatory cytokines/chemokines and also with ingravescence. Furthermore, anti-moesin pAb reacted with human neutrophils and monocytes to secrete inflammatory cytokines/chemokines *in vitro*. On the other hand, we need to investigate in a prospective study and follow-up study to increase the utility of the anti-moesin autoantibody in marker of vasculitis. The present study suggested, for the first time, that the anti-moesin autoantibody reacted with neutrophils and monocytes and is associated with the development of small vessel vasculitis.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://ndt.oxfordjournals.org.
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CONFLICT OF INTEREST STATEMENT

None declared.


REFERENCES

Metzincins and related genes in experimental renal ageing: towards a unifying fibrosis classifier across species

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ABSTRACT

Background. We have previously described a transcriptomic classifier consisting of metzincins and related genes (MARGS) discriminating kidneys and other organs with or without fibrosis from human biopsies. We now apply our MARGS-based algorithm to a rat model of age-associated interstitial renal fibrosis.

Methods. Untreated Fisher 344 rats (n = 76) were sacrificed between 2 to 104 weeks of age. For gene expression studies, we used single colour (Cy3) Agilent Whole Rat Genome 4 × 44k microarrays; 4–5 animals of each sex were processed at each of the following ages: 2, 5, 6, 8, 15, 21, 78 and 104 weeks. Intensity data were subjected to variance stabilization (www.Partek.com). Data were analysed with ANOVA and other statistical methods.

Results. Sixty MARGS were differentially expressed across age groups. More MARGS were differentially expressed in older males than in older females. Principal component analysis showed gene expression induced segregation of age groups by sex from 6 to 104 weeks of age. The expression level of MMP2 correlated best with fibrosis grade. Severity of fibrosis was determined in 20 animals at 78 and 104 weeks of age. Expression values of 15 of 19 genes of the original classifier present on the Agilent array, in conjunction with linear discriminant analysis, was sufficient to correctly classify these 20 samples into...