Expression of human 8-oxoguanine DNA glycosylase (*hOGG1*) in follicular lymphoma

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The human homologue of the yeast DNA repair enzyme 8-oxoguanine DNA glycosylase (*hOGG1*) repairs oxidatively damaged guanosine nucleotides in DNA. This enzyme is highly expressed in reactive germinal centers, where lymphoid cells are under oxidative stress, and has been thought to protect lymphocytes from mutation. As a first step to investigate the role of *hOGG1* in lymphomagenesis, we evaluated *hOGG1* expression in follicular lymphoma. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue of 28 follicular lymphoma cases (16 grade 1, seven grade 2, and five grade 3) to evaluate the expression of *hOGG1* in neoplastic follicles. Reactive germinal centers of non-neoplastic tonsil and lymph node tissue were also examined. Fluorescent-*in-situ* hybridization (FISH) was performed using a DNA probe from BAC clone RP11-266J6 corresponding to 3p25, where the *hOGG1* gene resides, to evaluate for the presence or absence of a deletion. In reactive germinal centers, the majority of centroblasts and centrocytes were positive for *hOGG1*. In contrast, the majority (21 of 28 or 75%) of follicular lymphoma cases showed absent/minimal expression of *hOGG1*. Only four of 28 (14%) follicular lymphoma cases revealed the same levels of *hOGG1* expression as reactive germinal centers. There was no correlation between *hOGG1* expression and histologic grade. None of the 16 cases evaluated by FISH showed a deletion of *hOGG1*. Furthermore, absent/minimal *hOGG1* expression was observed in four of six Bcl-2-negative follicular lymphoma cases. Our findings suggest that absent/minimal *hOGG1* expression occurs in the majority of follicular lymphomas. The downregulation of *hOGG1* does not appear to be due to a deletion of the *hOGG1* locus. Additionally, finding absent/minimal *hOGG1* expression in a subset of Bcl-2-negative follicular lymphomas suggests that *hOGG1* may have utility in diagnosing Bcl-2-negative follicular lymphomas.

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The human homologue of the yeast DNA repair enzyme, 8-oxoguanine-DNA glycosylase (*hOGG1*), excises 8-oxoguanine, an oxidative form of the guanine nucleotide.¹ *HOGG1* shares a 38% identity with the yeast *OGG1*. It has been localized to chromosome 3p25 by fluorescent-*in-situ* hybridization (FISH) by Radicella *et al* in 1997,² and it was cloned by Rosenquist *et al*³ in 1997. This DNA

glycosylase/apurinic lyase repairs oxidative DNA damage, specifically 8-oxoguanine/C base pairs. 8-hydroxyguanine is highly mutagenic via GC to TA transversions⁴ and is generated by oxidative stresses as well as normal cellular metabolism.⁵

It is believed that *hOGG1*'s role of excising 8-oxoguanine protects DNA against the mutagenic effects of oxidative damage and decreased cell survival from radiation-induced damage.⁶ Supporting *hOGG1*'s role of hindering mutagenesis is that certain a genetic polymorphism of *hOGG1*, Ser326Cys, has been implicated in an increased incidence of adenocarcinoma of the lung⁷ and squamous cell carcinoma of the lung^{8,9} in patients with the Cys/Cys genotype, as compared to Ser/Cys or Ser/Ser genotypes. Studies on oxidative damage and genetic polymorphisms of *hOGG1* have shown

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that *hOGG1* may play a role in solid tumor carcinogenesis including breast cancer,¹⁰ pancreatic adenocarcinoma,¹¹ and squamous cell carcinoma of the head and neck.¹² One recent review by Weiss *et al*¹³ indicates that there is epidemiologic evidence that *hOGG1* genotype may be related to risk of esophageal, head and neck, lung, nasopharyngeal, orolaryngeal, and prostate cancer. Oxidative damage and *hOGG1* expression has also been studied in non-neoplastic diseases. Iida *et al*¹⁴ have recently reported that decreased *hOGG1* expression associated with neurofibrillary tangles is observed in patients with Alzheimer's dementia suggesting a role for oxidative damage in the development of this disease.

A study by Kuo and Sklar demonstrated that *hOGG1* is highly expressed within germinal center B cells at levels greatly exceeding that found in other tissues, and that expression of this gene is most abundant within the dark, centroblast-rich zone of germinal centers.¹⁵ The expression of *hOGG1* in the dark zone of germinal centers may serve as a protective mechanism for the germinal center lymphocytes from oxidative damage-induced mutations arising as a result of processes such as antigen stimulation or release of oxygen-free radicals by macrophages in the germinal centers. To our knowledge, expression of *hOGG1* in lymphomas has not been studied.

The aim of this study was two-fold. The first aim was to evaluate the pattern of *hOGG1* expression in different grades of follicular lymphoma, a lymphoma of germinal center B-cell origin, and so doing, preliminarily evaluate the role of *hOGG1* in lymphomagenesis. The second aim was to use FISH to evaluate for the presence or absence of a deletion of the region containing *hOGG1* (3p25) and so doing, examine whether the mechanism for loss of *hOGG1* expression may be a deletion vs modulation of gene expression by some other mechanism.

Materials and methods

Patients

In all, 28 cases (13 males, 15 females; age range: 37–90 years old, mean 64 years old) of follicular lymphoma were retrieved from the files of the Department of Pathology, Medical College of Wisconsin, Milwaukee, WI, USA. The morphologic diagnosis of follicular lymphoma was reviewed and confirmed. Then, the cases were classified into grades 1, 2, and 3 according to criteria put forth by the WHO.¹⁶ The cohort of cases included 16 grade 1, seven grade 2, and five grade 3 follicular lymphomas. As a control non-neoplastic lymph nodes and tonsils were examined. The Medical College of Wisconsin Institutional Review Board approved the study protocol.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue was available in all of the cases. Paraffin sections were mounted on Fisherbrand/Plus Superfrost Pre-cleaned slides, heated at 60°C for 30 min, deparaffinized and rehydrated. Tissue sections were incubated with the rabbit polyclonal antibody to *hOGG1* (Novus Biologicals, Littleton, CO, USA) and Bcl-2 (clone: 124, Dako, Carpinteria, CA, USA) using the antigen retrieval techniques as described below. Slides were incubated for 30 min in 50:50 methanol peroxide then the antigen was retrieved in citrate buffer, pH 8.0 (*hOGG1*) or EDTA buffer, pH 10.0 (Bcl-2) for 30 min at 98°C then cooled for 20 min. Nonspecific protein-binding sites were blocked by serum for 5 min and the primary antibody added at different dilutions (1:50 for *hOGG1* and 1:100 for Bcl-2). All slides were stained using the Dako automated immunostainer (Dako, Carpinteria, CA, USA). The slides were incubated for 15 min in labeled streptavidin–biotin (LSAB) for linking and labeled subsequently with diaminobenzidine chromogen. Appropriate positive and negative controls were used in all cases.

Interpretation of Immunohistochemistry Results

As a control, reactive germinal centers of non-neoplastic tonsils and lymph nodes ($n=10$) were examined to assess the percentage of centroblasts and centrocytes positive for *hOGG1*. The percentage of either centroblasts or centrocytes positive for *hOGG1* was determined in a total of 1487 cells in 15 germinal centers. Morphologic criteria published by the WHO were used for identification of centroblasts and centrocytes.¹⁶

Expression of Bcl-2 and *hOGG1* was systematically reviewed by two of the authors (DM and CC) independently. The percentage of cells exhibiting positive staining for *hOGG1* and Bcl-2 was evaluated by examining 10 high-power fields ($\times 400$) in the follicular areas. The percentage of cells staining for *hOGG1* (nuclear staining) or Bcl-2 (cytoplasmic staining) in lymphoma cases were graded as follows: 0–5% = 0, 6–20% = 1+, 21–50% = 2+, >50% = 3+. Follicular lymphomas showing positive staining in $\leq 5\%$ of tumor cells were considered as having absent/minimal expression. The CD3 stain (highlighting the T cells) performed as part of the initial diagnostic workup in these cases was reviewed in conjunction with bcl-2 stain. The number of bcl-2-positive lymphoma cells was estimated by subtracting total CD3+ T cells from bcl-2-positive cells. Additionally, the bcl-2-positive lymphoma cells had lower staining intensity than bcl-2-positive T cells in most cases. This difference in staining intensity also allowed an accurate estimate of the percentage of bcl-2-positive lymphoma cells.

This grading system led to agreement between the two observers for over 95% of staining results. In the case of discrepant results, the two investigators re-evaluated the slide and eventually reached a consensus. The Bcl-2-positive T cells were used as a positive internal control to confirm that the antigens were retrievable for all the cases. Since *hOGG1* is only expressed in normal germinal center B cells, there was no positive internal control for *hOGG1* staining in the follicular lymphoma cases with absent *hOGG1* expression. However, appropriate controls were performed and evaluated with each staining run to ensure that the antibody was working correctly.

FISH Study for Determining the Copy Number of the *hOGG1* Gene

Preparation of probes

A search of the NCBI human genome project website identified the localization of *hOGG1* to chromosome 3p25 and BAC clone RP11-266J6. The clone was inoculated into 15 ml of LB broth medium (Q-Biogene, Carlsbad, CA, USA) containing 12.5 µg/ml of chloramphenicol and grown overnight at 37°C with shaking at 200 rpm. BAC DNA was extracted using the standard alkaline lysis protocol.

Labeling and reprecipitation of DNA probes

In all, 1 µg of BAC DNA was labeled with biotin-14-dUTP (Roche diagnostics, Indianapolis, IN, USA), using the standard Nick translation protocol. The labeled probe was resuspended in 10 µl of hybridization solution (50% formamide, 10% dextran sulfate, 2XSSC, dH₂O) and stored at -20°C until needed.

FISH

The probe was tested on known normal controls, using previously karyotyped peripheral blood lymphocytes, and metaphases were analyzed to verify hybridization to the appropriate chromosomal location. The FISH study was performed in 16 of 28 follicular lymphoma cases. Normal non-neoplastic tonsil was used as a negative control. Tissue sections (5 µm) were obtained on plus slides. Deparaffinization was accomplished using Citrusolv (Fisher Scientific, Pittsburgh, PA, USA) at room temperature for 10–15 min followed by air drying. The sections were dehydrated at room temperature in 75, 85, and 95% ethanol for 2 min each and were then air dried. The sections were treated with 2 × TissueDigest Solution (Insitus biotechnologies, Albuquerque, NM, USA) in a 37°C water bath for 2 h. Slides were dehydrated in an ethanol series again as mentioned previously. In all, 20 µl of a 50% glycerol/1 × SSC solution was placed over the tissue area and covered with a glass cover slip. The slides were baked in a 90°C oven for 5 min and allowed to cool. Slides were washed in 2 × SSC at room temperature for 2 min

followed by dehydration in 75, 85, and 95% ethanol at room temperature. After air drying, 5 µl of probe was applied to the tissue area, the slides were cover slipped and secured with rubber cement. The slides were placed in a humidified chamber (HYBrite, Vysis, Inc., Downers Grove, IL, USA), denatured at 80°C for 10 min, and hybridized overnight at 37°C.

After overnight hybridization, the slides were washed in 0.4 × SSC/0.3% NP-40 for 2 min at 72°C and then washed in 2 × SSC/0.1% NP-40 for 2 min at room temperature. Detection and amplification of the biotin signals were accomplished with incubation of the slides with FITC-avidin (Vector laboratories, Inc., Burlingame, CA, USA). The slides were counterstained with 15 µl of DAPI (Vector laboratories, Inc., Burlingame, CA, USA), cover slipped, and analyzed using a Zeiss Axiophot fluorescent microscope equipped with both single- and triple-band pass filters. Digital images were captured by a Power Macintosh G3 system and MacProbe version 4.0 (Applied Imaging, San Jose, CA, USA).

Scoring of signals

The slides were evaluated for the presence of signals. In all, 200 cells were scored for each case, looking for the presence of one, two, or more signals. A total of 400 cells were scored for normal non-neoplastic tonsillar tissue, and eight (2%) were found to have only one signal. Cases with more than 5% of cells exhibiting no or one signal were considered positive for a deletion, to increase specificity. Cases were photographed.

Results

In reactive tonsils and lymph nodes (*n* = 10), mantle zone B cells and interfollicular lymphocytes showed minimal *hOGG1* expression (≤5%), while germinal centers revealed high-level expression with 85% of centroblasts and 63% of centrocytes positive for *hOGG1* (Table 1 and Figure 1a and b). In contrast, the majority of follicular lymphoma cases (22/28 or 79% vs 0/10 of reactive lymph nodes or tonsils, *P* < 0.0001, Fisher's exact) showed absent or minimal (0–5%) staining of *hOGG1* in the neoplastic follicles (Table 2). These 22 cases with minimal or absent *hOGG1* expression included 12/16 grade 1, 6/7 grade 2, and 4/5 grade 3 follicular lymphomas. Two (both grade 1) of 28 cases (7%) showed low to moderate level *hOGG1* expression (1+ to 2+). Four

Table 1 Expression of *hOGG1* in normal tonsil and lymph node tissue

	Centroblasts	Centrocytes
Tonsils	90% (340/378)	65% (338/519)
Lymph nodes	72% (125/172)	60% (252/418)
Overall	85% (465/550)	63% (590/937)

Figure 1 Expression of *hOGG1* in reactive germinal centers and examples of *hOGG1* expression in follicular lymphoma cases. (a and b) *hOGG1* expression in a reactive germinal center with accentuated staining of the dark zone (a, $\times 200$) and with positive staining present in the nuclei of both centroblasts and centrocytes (b, $\times 400$); (c) negative staining for *hOGG1* by immunohistochemistry (IHC) in a grade 3 follicular lymphoma ($\times 400$); and (d) positive staining for *hOGG1* by IHC in a grade 1 follicular lymphoma ($\times 400$).

(two grade 1, one grade 2, and one grade 3) of 28 cases (14%) showed a high level of *hOGG1* expression (3+) comparable to reactive germinal centers. In general, cases with *hOGG1* expression showed strong intensity of staining. Examples of *hOGG1* expression in lymphoma cases are shown in Figure 1c and d. There was no correlation between the *hOGG1* expression status and the histologic grade of follicular lymphoma.

Most of the follicular lymphoma cases for which Bcl-2 data were available were bcl-2 positive as follows: 12/15 (80%) grade 1, 5/7 (71%) grade 2, 3/4 (75%) grade 3. Six of 26 cases (23%) showed absent staining of Bcl-2 (Table 2). These included three grade 1, two grade 2, and one grade 3 follicular lymphomas. Of those six cases, four (67%, two grade 1, one grade 2 and one grade 3) showed absent/minimal expression of *hOGG1*. One case (grade 1) showed a moderate level (2+) of *hOGG1* expression, and the remaining one (grade 2) showed a strong level (3+) of *hOGG1* expression. No correla-

tion between *hOGG1* expression and Bcl-2 expression was observed.

FISH for the *hOGG1* probe showed a normal dual green signal pattern in 196 of 200 (98%) cells scored in normal tonsillar tissue indicating that gene copy number could be reliably determined by FISH with tissue sections. Of 16 follicular lymphoma cases tested for *hOGG1*, four (25%, two grade 1, one grade 2, and one grade 3) showed positive *hOGG1* expression, and 12 (75%, four grade 1, four grade 2, and four grade 3) showed absent or minimal *hOGG1* expression. All cases showed a normal signal pattern (ie two copies of the *hOGG1* gene in each cell) in 97–100% of cells scored, indicating that no deletion was present (Table 2 and Figure 2).

Discussion

Our results indicate that downregulation of *hOGG1* expression at the protein level is a common feature

Table 2 Results of expression of Bcl-2 and *hOGG1* by immunohistochemistry and fluorescent *in situ* hybridization (FISH)

Case	Grade	<i>hOGG1</i> immunohistochemistry	Bcl-2	<i>hOGG1</i> FISH percent dual signal pattern (200 cells scored)	<i>hOGG1</i> FISH interpretation
1	1	1+ ^a	3+	100	No deletion
2	1	0	3+	97	No deletion
3	1	0	3+	ND ^b	ND
4	1	0	ND	100	No deletion
5	1	2+	0	98	No deletion
6	1	0	3+	100	No deletion
7	1	0	2+	100	No deletion
8	1	3+	3+	ND	ND
9	1	0	2+	ND	ND
10	1	0	0	ND	ND
11	1	0	0	ND	ND
12	1	0	3+	ND	ND
13	1	0	3+	ND	ND
14	1	3+	3+	ND	ND
15	1	0	3+	ND	ND
16	1	0	2+	ND	ND
17	2	3+	0	100	No deletion
18	2	0	3+	ND	ND
19	2	0	1+	99	No deletion
20	2	0	0	100	No deletion
21	2	0	3+	100	No deletion
22	2	0	3+	100	No deletion
23	2	0	3+	ND	ND
24	3	0	2+	100	No deletion
25	3	3+	ND	100	No deletion
26	3	0	3+	98	No deletion
27	3	0	0	99	No deletion
28	3	0	3+	99	No deletion

^aScoring system for *hOGG1* and Bcl-2 immunohistochemistry: ≤5% = 0 (negative), 6–20% = 1+, 21–50% = 2+, >50% = 3+.

^bND: not done.

Figure 2 An example of FISH for *hOGG1* in follicular lymphoma cases ($\times 1000$). In this case, two green signals are present and visible in >97% of cells indicating no deletion of the 3p25 locus.

for all histologic grades of follicular lymphoma. In this study, normal reactive germinal centers showed strong expression of *hOGG1* with increased expression of *hOGG1* in centroblasts (85%) compared to centrocytes (63%). This phenomenon is consistent

with the preferential expression of *hOGG1* in the dark or centroblast-rich zone of germinal centers as previously described by Kuo and Sklar¹⁵ who performed *in situ* hybridization analysis of GCN6 (*hOGG1*) expression with a cDNA probe on formalin-fixed paraffin-embedded sections of human tonsils. This confirms that the immunohistochemistry approach used in this study is reliable in determining the level of *hOGG1* expression in paraffin-embedded lymphoid tissues. We found that in contrast to the strong expression of *hOGG1* observed in non-neoplastic germinal centers, most follicular lymphomas of all histologic grades showed absent/decreased *hOGG1* expression. Only 4/28 (14%) cases had a level of *hOGG1* expression comparable to that of normal reactive germinal centers. Lymphoid cells in normal germinal centers are under oxidative stress due to antigen stimulation and may require increased expression of *hOGG1* to protect them from oxidative damage-induced mutation.

Thus, the downregulation of *hOGG1* expression in most cases of follicular lymphoma of all histologic grades suggests that there may be a predisposition to genetic mutations due to the accumulation of oxidatively damaged DNA in these cases. These mutations, particularly when involving important tumor suppressor genes or oncogenes, may subsequently lead to the development of follicular

lymphoma. It is unlikely, however, that downregulation of *hOGG1* by itself leads to the formation of follicular lymphoma since a recent study shows that somatic hypermutation of immunoglobulin genes, a key function of germinal center B cells, remain intact in the absence of *hOGG1* gene expression in knockout mice.¹ Also, these knockout mice do not have an increased rate of developing malignancy.^{1,17} Additionally, although the number of cases is relatively small in this study, *hOGG1* expression in follicular lymphoma did not correlate with disease stage at presentation, overall survival, or response to therapy (data not shown). Taken together, our findings suggest that downregulation of *hOGG1* expression may be an early event in lymphomagenesis. The exact role of *hOGG1* in the development of follicular lymphoma remains to be illustrated by further studies.

The mechanism leading to the downregulation of *hOGG1* expression in follicular lymphoma remains unclear. As none of the 16 cases tested by FISH showed a deletion of *hOGG1*, the mechanism for decreased expression of *hOGG1* in follicular lymphoma may be secondary to modulation of gene expression rather than deletion of the genetic locus. Our FISH results agree with previous cytogenetic studies indicating that abnormalities of 3p, where *hOGG1* resides, are rarely observed in follicular lymphomas, although complex numerical and structural karyotypic abnormalities, in addition to the t(14;18)(q32;q21), are typical of follicular lymphomas.^{18,19} We, however, cannot exclude the possibility that there may be a microdeletion of the *hOGG1* locus accounting for loss of expression of *hOGG1*. Since a BAC clone was chosen for this study, the size of a deletion detectable by this method is determined by the size of the BAC. BAC clones are typically 150–250 kb, and the length of BAC clone RP11-266J6 used in this study is 185.07 kb. Therefore, a microdeletion smaller than 185 kb would probably not be detected by this method. Smaller probes can be used; however, the sensitivity and specificity would decrease. Of note, Fan *et al*¹² have reported that majority (11/18 or 61%) of cases of head and neck squamous cell cancers show allelic loss of the *hOGG1* gene, using PCR-based analysis of two intragenic single nucleotide polymorphism sites, located in intron 4 and exon 7 of the gene. The deletion correlates with decreased *hOGG1* expression. However, this approach is only suitable for patients (40% or 18/45 in the study by Fan *et al*) with informative polymorphic loci of *hOGG1*. Alternatively, epigenetic changes, such as hypermethylation of promoter regions, may result in the decreased *hOGG1* expression. For example, hypermethylation of O⁶-methylguanine-DNA methyltransferase (MGMT), another DNA repair gene, leading to decreased MGMT expression has been shown in head and neck squamous cell cancers,²⁰ B-cell malignancies,²¹ and immunodeficiency-related lymphomas.²² Indeed, hypermethylation of MGMT

in lymphomas has been associated with chemosensitivity to alkylating agents and better survival.²³ However, preliminary data in a study by CY Fan *et al*²⁴ found that promoter hypermethylation of the *hOGG1* gene detected in 14 of 17 (82.3%) cases of head and neck squamous cell cancers did not correlate with decreased *hOGG1* mRNA levels. Further studies are required to illustrate the mechanisms leading to the downregulation of *hOGG1* in follicular lymphoma.

In addition to its possible role in the development of follicular lymphoma, *hOGG1* expression may have utility in diagnosing follicular lymphomas that are Bcl-2 negative. Bcl-2 has become an important marker to differentiate atypical reactive germinal centers (Bcl-2 negative) from neoplastic follicles in follicular lymphoma (Bcl-2 positive).²⁵ However, a small percentage of follicular lymphomas, particularly those that are grade 3, may lack immunohistochemical expression of Bcl-2 resulting in a diagnostic dilemma for pathologists.²⁶ The rate of Bcl-2 expression in grade 2 and 3 follicular lymphomas in this study is comparable with what has been reported previously in the literature with a larger number of cases by Lai *et al*.²⁷ However, the rate of bcl-2 expression is lower in grade I follicular lymphomas in the current study than the study by Lai *et al*.²⁷ This is likely due to sampling, as the number of cases in this study is relatively small compared to that by Lai *et al*.²⁷ The same clone and similar antigen-retrieval techniques were used in both studies.

In this study, there were six of 26 cases (23%) that lacked Bcl-2 expression. Four of these six cases (67%) were also negative for *hOGG1* expression. Of interest, these four cases included follicular lymphomas of all three grades. These findings suggest that absent/minimal *hOGG1* expression may be an important diagnostic marker for Bcl-2-negative follicular lymphomas since normal germinal centers strongly express *hOGG1*, as shown in the normal controls (tonsils and reactive lymph nodes). Further studies with more cases will be necessary to investigate *hOGG1*'s utility as a diagnostic tool for follicular lymphoma.

In summary, our findings suggest that decreased *hOGG1* expression occurs in the vast majority of follicular lymphomas. The downregulation of *hOGG1* expression does not appear to be due to a deletion of the *hOGG1* locus. Additionally, finding absent/minimal *hOGG1* expression in a subset of Bcl-2-negative follicular lymphomas suggests that *hOGG1* may have utility in diagnosing Bcl-2-negative follicular lymphomas.

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Duality of interest

None declared.

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